STUDIES ON TRANSCRIPTION IN THE GALACTOSE OPERON OF *E.COLI* K12.

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I. INTRODUCTION

A. The Operon and its Regulation

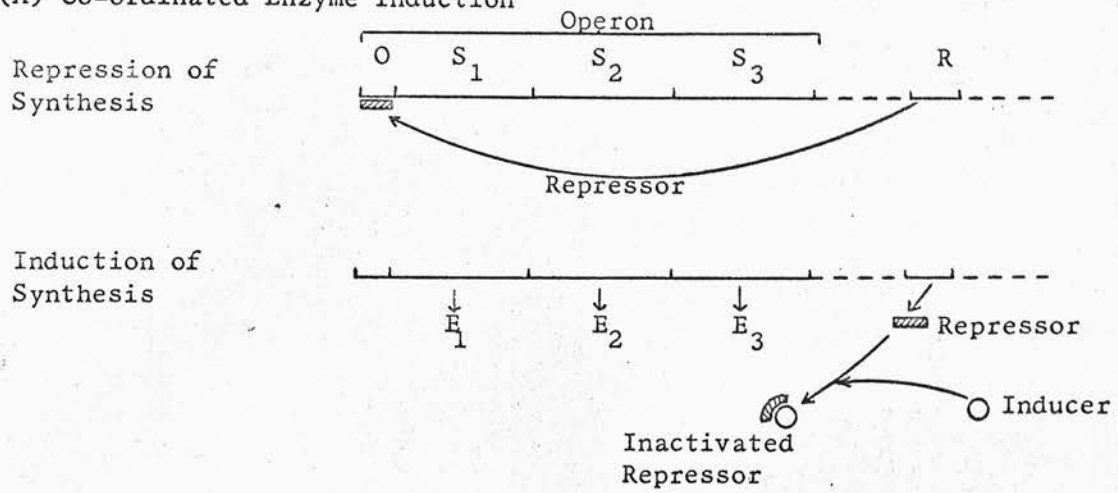
a) General Description

In bacteria the co-ordinate induction or repression of different enzymes in a single metabolic pathway is a common phenomenon. It was first observed for the enzymes of the arginine biosynthetic pathway by Vogel (1957) and Gorini and Maas (1958). Co-ordinate repression of the enzymes necessary for the utilisation of the substrates lactose and galactose was shown by Pardee et al (1959) and Kalckar et al (1959) respectively and of the enzymes in the tryptophan and histidine pathways by Cohen and Jacob (1959) and Ames and Garry (1959) respectively. Since then many other such systems have been demonstrated, including the arabinose (Engelsberg et al, 1965) and rhamnose (Power, 1967) catabolic pathways.

With the exception of the genes for the arginine biosynthetic enzymes, the genes for the different enzymes of a pathway have been shown to lie in a cluster on the genetic map. Such a cluster of genes, shown to be under a single genetic control, was named an operon by Jacob et al (1960).

A mechanism for the regulation of an operon was proposed by Jacob and Monod (1961a and b), and is outlined diagrammatically in figure 1. The regulator gene (R) produces a cytoplasmic repressor substance. In the case of inducible systems such as the galactose and lactose operons this repressor substance interacts in some way with the operator gene (O) thus preventing the expression of the operon. Addition of the inducing substrate

(A) Co-ordinated Enzyme Induction



(B) Co-ordinated Enzyme Repression

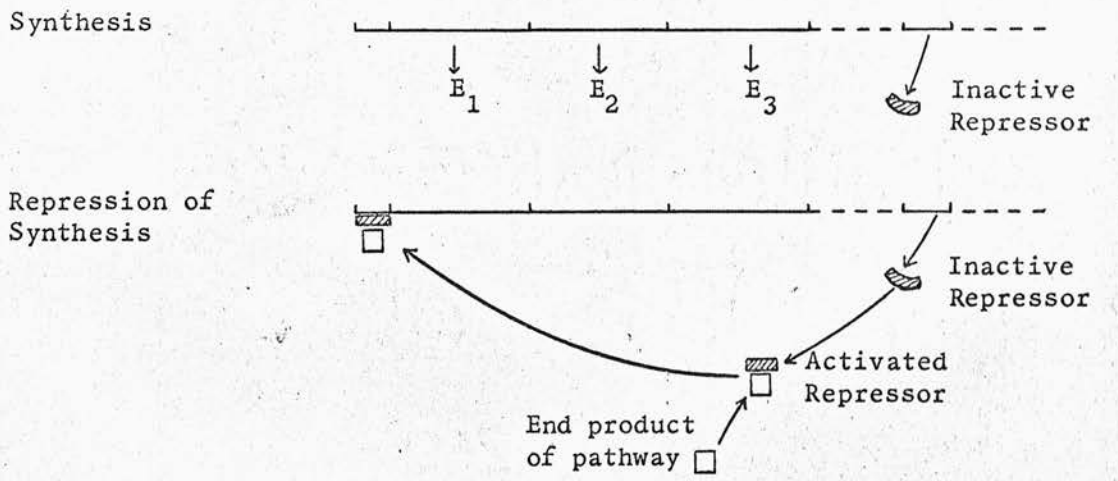


FIGURE 1. Regulation of the operon.

Abbreviations : S_1, S_2, S_3, genes specifying structural proteins; O, operator gene, R, regulator gene specifying active(Figure A) or inactive (Figure B) repressor; E_1, E_2, E_3, structural proteins.

prevents the repressor-operator interaction by binding between the substrate and the repressor and hence enables the operon to function (see figure 1A). In the case of repressible systems such as the tryptophan and histidine operons the presence of the amino acid end-product causes repression of the biosynthetic enzymes. Here a slightly different mechanism is believed to operate: The binding of repressor to the end-product of the pathway allows repressor-operator interaction and removal of end-product results in the operon being freed from repression (figure 1B).

This model was proposed to explain negatively controlled systems. Positively controlled systems will not be discussed here.

The existence of the various components of the operon as originally defined, and its regulatory gene were first demonstrated genetically in the lactose operon (Pardee et al, 1959) and are shown in figure 2. The existence of the regulator gene was shown by two types of mutations which map outwith the operon region. The first of these is the constitutive mutation which allows the synthesis of all the gene products of the operon whether or not inducer is present. These mutations, designated i^-, were shown to be recessive to the wild type (i^+) when the two were brought together in heterogenotes. The second type of mutation is the super-repressor (i^S) which permanently prevents the utilization of lactose. The i^S mutation is dominant over wild type (Jacob and Monod, 1961a; Willson et al, 1964).

The operator region was also defined genetically by mutations which produce the same phenotypes, i.e. constitutive (O^c) and operator

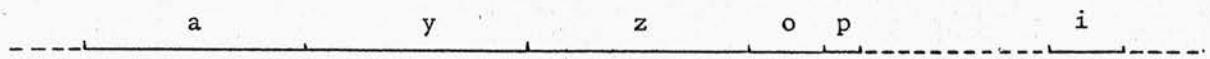


FIGURE 2. Genetic map of the lactose operon of E.coli

Symbols	Type of locus	Product
a)	Structural genes	galactoside transacetylase
y)		galactoside permease
z)		β-galactosidase
o	operator	-
p	promotor	-
i	regulator	repressor

negative (O^o) mutations. The O mutations were shown to map at the extreme end of the Z gene, and to function only in the cis position (Jacob et al, 1960). Since then, however, the O^o class have been shown to be mutations in the structural gene, Z, which exert a polar effect on the whole operon. (Beckwith, 1963; Beckwith and Brenner, 1965). Polar mutants will be discussed in more detail later.

That the operator is distinct from the Z gene has been demonstrated by the isolation of deletions which give an O^c phenotype but do not affect β-galactosidase activity. (Jacob et al, 1964). ~~Fine genetic analysis of the region using 4-factor crosses confirmed this. (Jacob and Monod, 1964).~~

In the original hypothesis the operator was assigned two functions, as the site both of repressor action and of DNA dependent RNA polymerase attachment. Since then another element the promotor region has been postulated as the site of polymerase attachment and thus of initiation of transcription. (Jacob et al, 1964). This was necessary to explain the finding that O^c deletion mutants fell into only three of the four expected classes. These were deletions of O itself; of i and O; and of i, O and Z. None were found which involved only O and Z, and this was interpreted to mean that deletions of this type were inviable due to the presence of a controlling element in the region between Z and O. Scaife and Beckwith (1966) isolated mutants which gave a reduced maximal level of synthesis of all the enzymes but which were still under the control of the i gene

i.e. with the property expected of mutations in a region controlling initiation of transcription. They mapped these between O and Z. However later work (Miller et al., 1968; Ippen et al., 1968) has located the promotor region on the other side of O, ~~i.e. on the side suggested by the initial study of Jacob et al. (1964)~~.

b) The Repressor.

The presence of a cytoplasmic repressor substance causing repression of an operon is indicated by the response of the operon to a regulator gene in the trans configuration. In the lactose operon evidence ~~also~~ comes from crosses involving constitutive and wild type regulator genes. If the recipient carries the recessive constitutive allele it is found that the enzymes are synthesised, in the absence of inducer, for approximately one hour after mating is interrupted. In the reciprocal cross no synthesis occurs in the absence of inducer (Pardee et al., 1959). Thus the recipient cytoplasm is important in determining the initial phenotype of the heterogenote. A similar phenomenon is found in the zygotic induction of phage λ .

The repressor was initially thought not to be a protein since addition of inhibitors of protein synthesis did not prevent the expression of the lactose wild type regulator genes on entry into a constitutive cell (Pardee and Prestidge, 1959). In 1965, however, suppressible nonsense mutations of the lac regulator gene were isolated (Bourgeois et al.). Such mutations are known to act at the level of translation of mRNA into

protein (Beckwith, 1963; Brenner and Beckwith, 1965) which suggested that the repressor was, in fact, protein.

The lac repressor has since been isolated and partially purified by Gilbert and Müller-Hill (1966). They were able to identify from wild type cells a fraction of cellular protein which specifically binds the gratuitous inducer isopropylthiogalactoside (IPTG). This fraction was shown to be absent in regulator constitutive mutants. The binding was shown to be inhibited by pronase but not by RNAse or DNAse. Gilbert and Müller-Hill (1967) and Riggs et al (1968) showed that this lac repressor specifically bound to DNA containing the lac operator and addition of inducer prevented this. The repressor was finally purified and characterised as protein by Riggs and Bourgeois (1968). They could detect no nucleic acid component.

Similarly, Ptashne (1967a) isolated a protein fraction from cells infected with phage λ under conditions which allowed the synthesis only of the λ repressor substance. This fraction was shown to be absent in cells infected with phage carrying an amber mutant in the λ regulator gene and to have different properties when isolated from cells infected with a temperature sensitive mutant of the regulator gene. He further showed (1967b) that the repressor fraction bound to λ^+ DNA 'in vitro' but not to a mutant, λ imm^{434} DNA which carries the regulator gene of phage 434.

The likelihood of protein repressors being a general phenomenon is feasible since each operon has its own specific repressor molecule and protein molecules could best achieve the range of specificities required.

B. Polar Mutations

In all the operons studied to date, pleiotropic mutations have been isolated. These have been termed polar mutations and can occur in all but the operator distal cistron of the operon. Their pleiotropic effect extends mainly to those genes on the operator-distal side of the mutation although the phenomenon of antipolarity (i.e. reduction of expression of genes proximal to the mutation) has been observed.

A gradient of polarity has been demonstrated with individual genes of most operons, the mutations with the strongest polar effect being found at the operator-proximal end. By constructing double mutants of the lac operon comprising a polar nonsense mutation and a deletion, Newton (1966) was able to examine the strength of polarity relative to position of the polar mutation within the gene. He demonstrated that the strength of polarity was a function of the distance between the mutated site and the operator distal end of the cistron.

Four types of mutations are known to cause polarity. These are nonsense mutations, deletions, insertions and frameshifts.

Nonsense mutations cause the termination of peptide chain synthesis at the site corresponding to the mutated site on the DNA. (Stretton and

Brenner, 1965). Three types of nonsense mutations are now known, amber (Epstein et al, 1963), ochre (Beckwith, 1963), and UGA (Brenner et al, 1967).

In the presence of an appropriate suppressor (su^+) allele it has been shown that a different amino acid replaces the normal one at the mutated site in the peptide chain. (See Stretton et al, 1966 for a review of ochre and amber su^+ allele replacements).

Engelhardt et al (1965) and Capecchi and Gussin (1965) showed that tRNA from an su^+ strain of bacteria could suppress amber mutations 'in vitro'. Likewise, Smith et al (1966) showed that RNA prepared from strains of bacteria carrying the su^+_{III} allele contained a tyrosyl-tRNA which recognised the amber codon but not the normal tyrosine codons. This specific tRNA was absent in RNA from su^- bacteria.

It therefore seems that the su^+ alleles produce different kinds of tRNA molecules from the su^- alleles. The anticodons of the su^+ tRNA molecules presumably recognise their corresponding nonsense triplets and insert an amino acid in this position whereas su^- tRNA's do not recognise them, and thus translation is stopped in su^- cells.

Frameshift mutations (Crick et al, 1961) are believed to be caused by the insertion or deletion of one or two base pairs, so that the triplet code thereafter is read out of phase. The polarity effect caused by frameshifts can result from the formation of a nonsense codon in the altered base sequence. Alternatively, it is possible that a vastly

altered and inactive protein is translated from the altered DNA sequence.

Deletions and insertions are terms given to mutations involving a larger segment of the DNA than do frameshifts. They could also, however, produce a shift in the reading frame with the same consequences. Insertions will be discussed in more detail in the section on polar mutations in the gal operon.

C. Transcription of the Operon

a) Polycistronic mRNA

The most simple mechanism which would allow one operator to control the expression of several genes is the ~~transcription~~ synthesis of the mRNA in a polycistronic form.

Evidence for this has been reported for the lac operon. By comparing the sedimentation of RNA from induced and non-induced lac^+ bacteria Guttman and Novick (1963) found an increase in the fraction corresponding to 30S in the induced preparation. Attardi et al (1963b) eluted RNA preparations from a methylated albumin column and found that mRNA produced on induction of the lac operon eluted before the 23S RNA. Since crude calculations based on the molecular weight of a single protein of the lac operon results in an expected mRNA fraction of less than 16S it appears that the lac mRNA is polycistronic.

Similarly mRNA isolated from the histidine operon of Salmonella by Martin (1963) was shown to have a sedimentation value of 34S which agrees

well with the predicted 38S if all the histidine genes are transcribed into a single molecule. (Martin and Ames, 1961; Hartman quoted by Martin, 1963).

A multicistronic mRNA molecule has also been demonstrated for the tryptophan operon (Imamoto et al., 1965). RNA isolated from derepressed wild type cells and hybridised to specific DNA was shown to be 33S whilst RNA's from trp deletion mutants were found to be smaller.

b) mRNA Levels under Different Physiological Conditions.

Jacob and Monod (1961a and b) reported an increase in the level of fast labelling RNA on induction of the wild type lac operon. No increase was found on induction of an O^{o} mutant. As already mentioned Guttman and Novick (1963) also found an increase in a specific fraction of RNA on induction of the lac operon. In both cases, however, the specificity of this RNA was not tested.

Hayashi et al.(1963) using the hybridization technique of Hall and Spiegelman (1961) showed that RNA from an induced culture of lac^{+} cells formed more hybrid with DNA from a defective phage Pldl carrying the lactose operon than did RNA from a non-induced culture. Using that fraction of the RNA which showed an increase on induction they found that the ratio of induced to non-induced hybridization increased from 8 with total RNA to 30.

Lac specific mRNA was also shown to increase on induction by Attardi et al.(1963a and b). They used an F-lac element as the source of

lac DNA and hybridization was carried out by DNA-agar gel column chromatography (Bolton and McCarthy, 1962). They found a 13-fold increase in hybrid formed after induction.

In the trp operon Imamoto et al. (1965a and b; 1966) have demonstrated the presence of a trp specific mRNA synthesised on derepression of the operon. This was detected by hybridization of RNA to DNA from the defective phage Ø80 pt carrying part or all of the trp operon.

The transcription of this operon has been shown to be periodic (Imamoto, 1968; Baker and Yanofsky, 1968) suggesting that the initiation of polymerase activity, at least in this case, is under strict control. The mode of action of this control has yet to be determined.

There is, therefore, good evidence that specific mRNA is synthesised on induction of the lac operon and derepression of the trp operon. This, however, does not necessarily mean that the regulation occurs at the level of transcription. Any method of coupling the regulation of transcription to that of translation could place the primary reaction at the level of translation. That this apparently occurs at least in one situation has been demonstrated with nonsense mutations.

c) mRNA Levels in Nonsense Mutants.

It is clear that the nonsense mutations act at the level of translation, yet there is evidence that a reduced level of mRNA may be ~~transcribed~~ synthesised in su^- cells carrying a nonsense mutation.

Attardi et al (1963b) carried out hybridization experiments with RNA prepared from O^{o} mutants of the lactose operon which were subsequently found to be nonsense mutants (Beckwith, 1963; Brenner and Beckwith, 1965). They showed that the level of hybridization of the mutant RNA to F-lac DNA was comparable to non-induced wild type RNA and this level was not increased after induction.

By comparison of hybridization saturation curves of various polar mutants of the z and y genes of the lac operon, Contesse et al (1966) demonstrated that the amount of hybrid formed depended on the distance of the mutated site from the beginning of the operon. The more operator distal mutations gave higher saturation values. They concluded that the lower hybridization values obtained with polar mutant RNA's is due to the absence of RNA beyond the mutated site.

Likewise Imamoto et al (1966) showed that RNA from nonsense mutants of the trp operon gave low hybridization with Ø80dt DNA, and this level was increased in the presence of an appropriate suppressor gene. These authors further showed that this reduction was due to the lack of mRNA corresponding to the genes distal to the mutated site on the DNA.

These data can be interpreted in three ways (Imamoto et al, 1966) namely: Very little mRNA is ~~transcribed~~ synthesised beyond the point of the nonsense mutation; the mRNA is ~~transcribed~~ synthesised 'in toto' but only rarely is the mutation-distal portion released from the DNA, i.e. during translation; the

mRNA which is not being translated is rapidly degraded.

Evidence that RNA from nonsense mutants is more rapidly degraded than wild type has been provided by the kinetic hybridization studies of Morse and Yanofsky (1969) and Morse and Primakoff (1970) with nonsense mutants of the tryptophan operon of E.coli.

D. The Galactose Operon and its Control

(a) General Description

The co-ordinate induction of certain enzymes involved in galactose utilisation was first shown by Kalckar et al.(1959). The reactions involving these enzymes, galactokinase (kinase), galactose-1-phosphate uridyl transferase (transferase) and uridine diphosphate galactose-4-epimerase (epimerase) are shown in figure 3 (Kurahashi, 1957; Kalckar et al., 1959).

It was subsequently shown by Buttin (1961, 1963a) that they were also induced co-ordinately by the gratuitous inducer D-fucose and co-ordinately repressed by β-D-thiogalactosides. Since D-fucose is not metabolised by these enzymes it was apparent that the induction of the enzymes was not sequential, i.e. not occurring as substrate became available.

Lederberg (1960) demonstrated that four of the five known complementation groups found amongst gal$^-$ mutants (Morse et al., 1956a and b) could be transduced by phage λ and were thus closely linked on the genetic

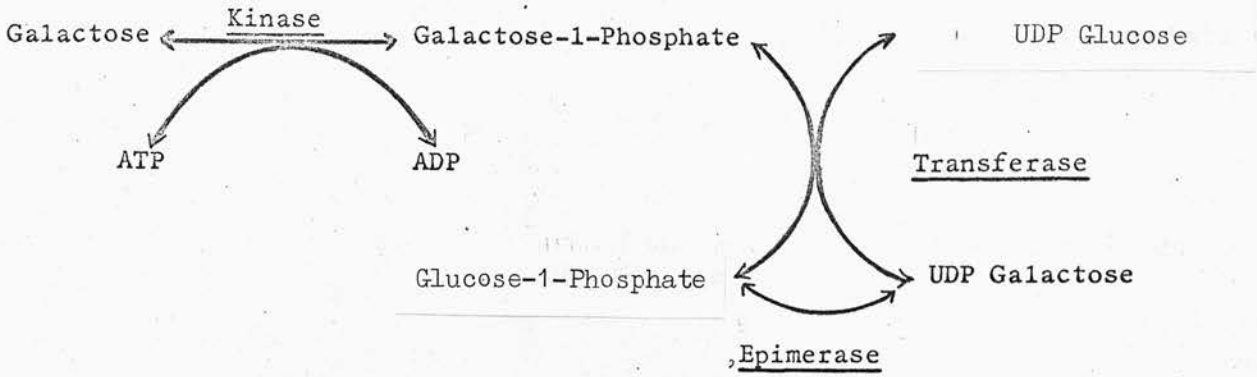


FIGURE 3. Galactose utilisation in E.coli.

Abbreviations: ATP adenosinetriphosphate; ADP adenosinediphosphate; UDP Galactose, uridine diphosphategalactose; UDP Glucose, uridine diphosphateglucose.

map. Mutations in three of these groups had been shown previously to have defects in the kinase, transferase and epimerase enzymes respectively. (Kurahashi, 1957; Kalckar et al., 1959; Soffer, 1961). The fourth group was defective in all three enzymes and will be discussed later.

b) Genetic Map of the Galactose Operon and its Regulator Gene.

The order of the gal structural genes has been established to be k-t-e using various fine structure mapping techniques to determine the order of several mutations (figure 4) (Morse, 1962; Adler and Kaiser, 1963; Buttin, 1963b; Shapiro, 1967; Adhya and Shapiro, 1969). Its orientation in relation to the prophage λ was demonstrated by Adler and Templeton (1963) and Shapiro and Adhya (1969), using a series of bacterial deletion mutants in crosses with gal^- mutants, to be k-t-e-λ. These deletions extended from within the prophage to various points within the gal genes.

That these genes form an operon as defined by Jacob and Monod (1961a and b) was first demonstrated by Buttin (1961, 1962, 1963b). He isolated mutants which synthesised the three enzymes constitutively. Three of these were studied in detail and found to fall into two categories.

One constitutive mutant was typical of an operator constitutive (O^c) type. Co-transduction of this mutant and the structural gal genes by phage λ showed that it was closely linked to this region. Furthermore it was shown to exert its effect only in the cis position.

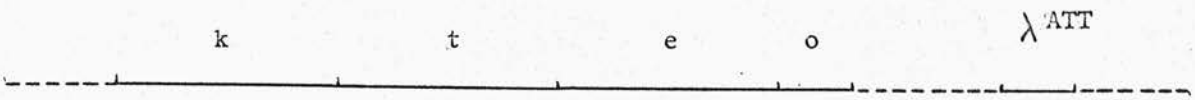


FIGURE 4. Genetic map of the galactose operon of E.coli.

Symbols	Type of locus	Product
k)		Galactokinase
t)	Structural genes	Galactose-1-phosphate uridyl transferase
c)		Uridine diphosphogalactose-4-epimerase
o	Operon	-
λ ATT	Site of attachment of prophage λ	-

The position of the operator was shown by Buttin (1963b) to be closest to the e gene, farther from the t and farthest from the k cistron.

Further evidence placing the operator at the epimerase end of the gal region has come from several sources. Michaelis and Saedler (1967) showed the sequential appearance of the epimerase, transferase and kinase enzymes in that order. Guha et al.(1968) demonstrated that transcription of the gal operon started at the epimerase gene by hybridization techniques which will be discussed in more detail in a later section. Finally Shapiro and Adhya (1969) demonstrated the existence of polar mutants in both the e and t genes affecting transferase and kinase, and kinase activity respectively but none in the k gene affecting the activity of the other two genes. This and their evidence that deletions extending from prophage λ into the epimerase gene impair t and k functioning confirms the order of the gal cistrons and operator shown in figure 4.

Buttin's other two constitutive mutants were identified as having mutations in a region unlinked to the gal operon mapping close to the lysine marker. The locus defined by these mutations was termed R gal and corresponds to the i gene of the lac operon. Saedler et al.(1968) isolated non-inducible gal mutants (R^Sgal) which mapped in the same region thus confirming the nature of the locus.

By the construction of heterogenotes, Saedler et al.(1968)

demonstrated that their R^S gal mutants were dominant over the wild type allele which in turn is dominant over the constitutive mutation. Furthermore, revertants of their R^S gal mutants and deletions extending into the mutated gene were found to be constitutive which strongly suggests that like the lac operon, gal is under negative control.

E. Transcription of the Galactose Operon

The induction of gal mRNA has been demonstrated by Attardi et al (1962, 1963 a and b), Hill and Echols (1966) and Guha et al. (1968). In all cases hybridization techniques were used with RNA from cells grown in the presence or absence of fucose and DNA from λ dg a defective phage λ carrying the complete gal operon. In all cases DNA was in excess.

Attardi et al. took 8-10S RNA purified on a sucrose gradient, from labelled induced and non-induced cultures of wild type cells. This was mixed with heat denatured DNA from λ^+ or λ dg phages and incubated to allow hybridization. The hybrids were separated from free RNA by CsCl gradient centrifugation and the radioactivity in the hybrid counted. After correction for hybridization to the λ^+ component the λ dg they found a difference of 6.5-fold between induced and non-induced preparations.

Hill and Echols (1966) used a hybridization technique based on that of Nygaard and Hall (1963) with the additional step of RNAse treatment before sampling on the nitrocellulose membranes. They found a 3-fold increase in the amount hybrid formed with an induced RNA preparation.

The result of this work, however, differs from that of Attardi et al, in that Hill and Echols observed only 17% cross hybridisation with λ^+ DNA whereas Attardi et al found 40%. This and the different quantitative increase in gal RNA on induction may, however, be accounted for by the fact that Attardi et al used a specific fraction of RNA. An increase in the difference between induced and non-induced RNA hybridization was found by Hayashi et al. (1963) on using a specific lac RNA fraction.

Guha et al. (1968) carried out hybridization experiments according to the method of Gillespie and Spiegelman (1965). They found an average increase of 2.4-fold (varying from 2- to 9-fold)of gal specific RNA on induction. By separating the strands of the λ dg DNA and hybridizing RNA to each singly they found an increase on induction of 5-fold in the hybrid formed with the lighter strand. No increase was found with the denser strand. Thus gal mRNA is ~~transcribed~~ synthesised from the lighter strand, and since the 3' end of this is at the epimerase cistron end of the gal operon the order of transcription must be e-t-k. As already mentioned, this places the operator at the epimerase end of the operon.

Attardi et al. (1963a and b) claimed a quantitative correlation between mRNA and enzyme activity levels in the gal operon under different physiological conditions. A closer examination of their data, however, suggests this to be optimistic. Under non-induced conditions the gal

mRNA was found to be 6.5 times less than under induced conditions whereas the enzyme difference was 15-fold.

Similarly, Hill and Echols (1966) found that a 10-15-fold increase in enzyme activity was accompanied by a 3-fold increase in gal mRNA level.

Since regulation at the level of transcription should result in a quantitatively correlated increase on induction of enzyme activity and mRNA level we decided to investigate transcription of the gal operon in more detail, and attempt to examine its regulation. In so doing, a method of analysis has been developed (Bishop, unpublished work) which makes the evaluation of the hybridization data more meaningful.

Two types of hybridization experiments were carried out. 1) Saturation experiments, in which the amount of DNA was kept relatively constant and increasing amounts of RNA were added. 2) Competition experiments in which a fixed amount of radioactively labelled RNA was diluted with increasing amounts of cold RNA preparations.

A λ dg carrying the complete gal operon and a λ dg carrying only the epimerase gene were used as sources of gal DNA. λ^{+} DNA was used as a control for cross-hybridization between this and E.coli RNA.

Preparations of RNA were made from E.coli K12 carrying an F^{1} gal element in addition to the gal operon on the bacterial genome.

This increases the proportion of gal genes in the cells to approximately 2.5 times that of cells without the F^1 gal. The gal operon was induced with D-fucose or repressed with T.M.G. in order to compare the hybridizible fraction under the different physiological conditions.

F. Polar Mutations of the Galactose Operon

Of the polar mutants isolated in the gal operon two main classes have been identified. These are nonsense mutants (Adhya and Shapiro, 1969) and insertion mutants (Jordan, Saedler and Starlinger, 1968; Shapiro, 1969).

Many nonsense mutations were studied by Adhya and Shapiro (1969). These were shown to map throughout the transferase and epimerase genes (Shapiro and Adhya, 1969) and, as in other operons, a gradient of the polar effect is found within each gene (Shapiro and Adhya, 1969).

By comparison of buoyant densities of λ dg's carrying the mutations to λ dg gal^+ and to their own revertants, Jordan, Saedler and Starlinger (1968) showed that two extremely polar transferase mutants and the epimerase polar mutant, gal^-q of Lederberg (1960) were due to an insertion of genetic material. Similarly four other extreme transferase polar mutants were demonstrated to be due to an insertion by Shapiro (1969).

All these mutants had previously been shown to produce low levels of enzyme, to be insensitive to nonsense suppressors, to revert spontaneously at a low frequency and to be insensitive to base analogue and frameshift

mutagens. Several other polar mutants of the gal operon have these properties (Lederberg, 1960; Shapiro, 1967; Adhya and Shapiro, 1969; Saedler and Starlinger, 1965, 1967a and b) and may also belong to this category.

Of particular interest amongst the gal polar mutants with the properties of an insertion mutation is gal$^-$3 (Lederberg, 1960). It has been suggested that this mutation may involve a controlling element in the galactose operon - possibly the promotor site - due to its anomalous properties: The basal level of kinase and transferase in gal$_3^-$ cells is only very slightly increased on induction by fucose (Hill and Echols, 1966; Bishop, unpublished work). Revertants of gal$_3^-$ fall into three classes which include constitutive cells synthesising the enzymes at the normal, induced level (Hill and Echols, 1966) and cells which produce the enzymes at a higher level than non-induced wild type but which are non-inducible (Morse, 1967) or very poorly induced (Hill and Echols, 1966) as well as normal inducible revertants (Hill and Echols, 1966; Morse, 1967)

Since Shapiro and Adhya (1969) mapped gal$_3^-$ at the operator proximal end of the gal operon without being able to distinguish between the e gene and a controlling element before it, it is feasible that this mutation is in the controlling element.

Many other polar mutants with properties compatible with their being in a controlling element have been reported (Saedler and Starlinger, 1967a and b; Adhya and Shapiro, 1969) and some of these mapped in the

same region as gal_3^- (Shapiro and Adhya, 1969) but will not be discussed in detail here. It must be pointed out, however, that several of these, like gal_3^-, appear to have a twofold effect on basal level of enzyme synthesis and on induction property. This suggests that the operator/promotor region in gal is one unit (unlike the lac operon) or as suggested by Saedler and Starlinger (1967b) these mutations are caused by an inversion involving both controlling elements.

Three extreme epimerase polar mutants have been isolated in this laboratory, NA 119, NA 121, NA 156. All three produce low basal levels of transferase and kinase and are inducible. The levels of enzymes produced by these mutants are shown in table 5.

It was decided to examine the level of mRNA in the mutants gal_3^-, gal_9^-, NA 119, NA 121 and NA 156 under induced conditions and to examine this in relation to the level of enzyme activity.

A basic genetic analysis was also carried out in an attempt to find the nature of the mutations.

II. MATERIALS AND METHODS

A. Bacteria and Bacteriophage Strains

The bacteria and phages used in this work are listed with their genotypes in tables 1, 2, 3 and 4. Unless otherwise stated the strains used were isolated in this laboratory by Dr. J.O. Bishop, Dr. J.N. Davison and Mr. R. Frame.

The positions of the polar mutations used are shown in figure 5. Using Dr. J. Shapiro's e deletions, mutants NA 119, NA 121, NA 156, Gal$^-$3 and Gal$^-$9 were mapped in this laboratory by Dr. J.O. Bishop. Gal$^-$3 and Gal$^-$9 were also mapped by Dr. J. Shapiro (1967).

The levels of gal enzyme activities in the induced polar mutants expressed as a percentage of the wild type induced level are given in table 5.

The lengths of F^{1}8, λ dg 17-8, λ dg T166 and the extent of the deletion, 9-3, are shown in figure 6.

B. Media

1) M.S. Broth (Davies and Sinsheimer, 1963) contains per litre, 10g Difco Bacto Tryptone, 8g NaCl, and 1g Difco Bacto Yeast Extract. To the above mixture immediately before use was added per litre, sterile 10% glucose, 10 ml; 1M $CaCl_2$, 2 ml; and 10% $MgSO_4.7H_2O$, 2ml.

TABLE 2. Female Stocks of Bacteria

Bacterial Symbol	General Genotype	Gal Genotype	Gal Phenotype	Source
9-3	$His^- trp^- gal^- Sm^r su^- \lambda^s$	Complete deletion	-	
9-3h	$His^+ trp^- gal^- Sm^r su^- \lambda^s$	Complete deletion	-	
9-3t	$His^- trp^+ gal^- Sm^r su^- \lambda^s$	Complete deletion	-	
9-2	$His^- trp^- gal^- Sm^r su^- \lambda^s$	Deletion of e, t and part of k.	-	
E1	$His^- trp^- gal^+ Sm^r su^- \lambda^s$	$k^+ t^+ e^+ pp^+$	+	
A22	$Gal^- \lambda^s Sm^s$	$k^+ t^+ e^- pp^+$	-	Adler
A24	$Gal^- Sm^s \lambda^s$	$k^- t^+ e^+ pp^+$	-	Adler
$AB1157_{A8}$	$Trp^-_{A8} Pro^- Thr^- Leu^- His^- Thi^- Arg^- Lac^- Gal^- Man^- Xyl^-$ $T_1^s T_6^r Sm^r \lambda^s Rec^+ su^-$	?	-	Brammar
$AB1157_{A21}$	$Trp^-_{A21} Pro^- Thr^- Leu^- His^- Thi^- Arg^- Lac^- Gal^- Man^- Xyl^-$ $T_1^s T_6^r Sm^r \lambda^s Rec^+ su^-$	?	-	Brammar

TABLE 3. Stocks of Lysogenic Bacteria

Bacterial Symbols	Genotype
E1(857)/λ	As E1 (Table 2) λ^{-r} lysogenic for the heat sensitive phage λ^{857}.
17-8/λ	As A22 (Table 2) λ^{r} doubly lysogenic for λ^{857} and λ^{857} dg 17-8 which carries the complete gal operon.
T166/λ	As A22 (Table 2) λ^{r} doubly lysogenic for λ^{857} and λ^{857} dg T166 which carries the epimerase gene of the gal operon.

TABLE 4. Bacteriophage Strains

Phage	Genotype
λ^+	Wild type
λ^v	Virulent strain of λ
$R_{II}1272$	T_4 carrying complete deletion of RII region.
f2	Wild type
T_4	Wild type
Am 272) Am 17)	T_4 head protein amber mutants suppressible by amber suppressors only.
Am 22	T_4 head protein amber mutant suppressible by amber and ochre suppressors only.
λ dg 17-8	λ defective carrying the complete gal operon (see figure 6)
λ dg T166	λ defective carrying the epimerase gene of the gal operon (see figure 6).

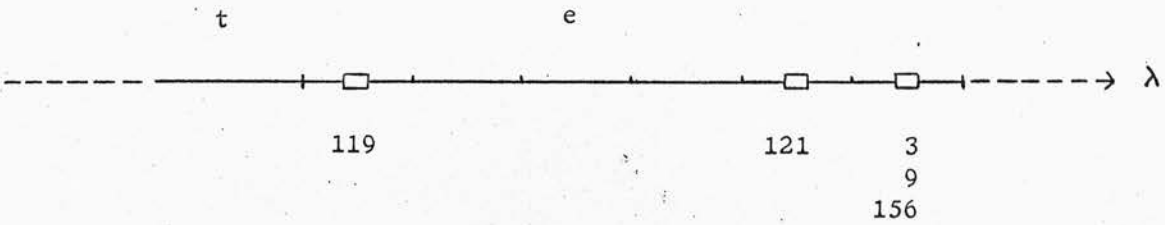
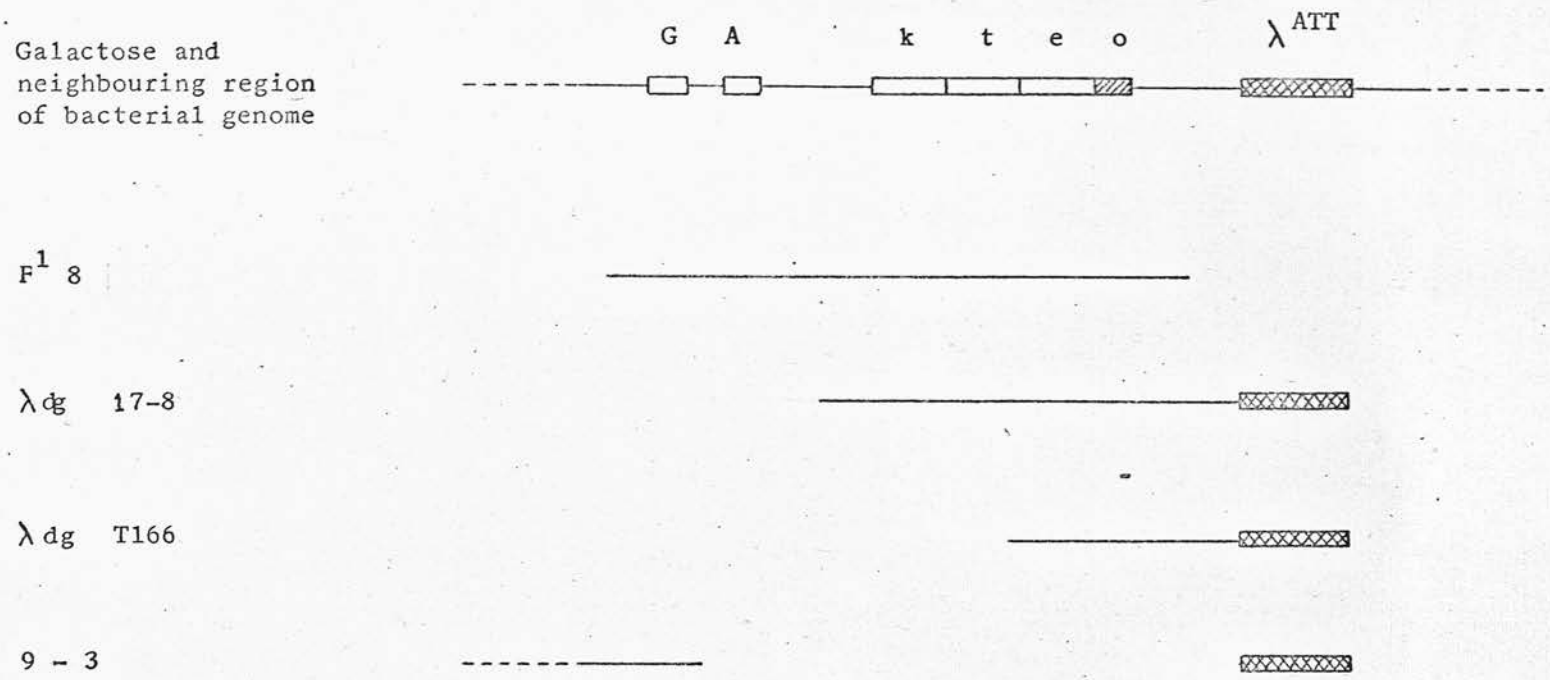


FIGURE 5. Position of mutations Gal⁻3, Gal⁻9, n119, n121, 156, within the epimerase gene of galactose operon.

TABLE 5. Levels of Galactose Enzymes in Various Bacterial Strains Compared with E1/F8 Induced Level.

Bacteria	Growth Conditions	Enzyme levels expressed as percentage of E1/F8 Induced Level
E1/F8	Repressed with TMG	3.3
E1	Induced with D-Fucose	40.0
NA119/119	Induced with D-Fucose	20.0
NA121/121	Induced with D-Fucose	10.0
NA156/156	Induced with D-Fucose	5.0
F Gal$^-$3	Induced with D-Fucose	< 1.0
F Gal$^-$9	Induced with D-Fucose	< 1.0



Symbols	Locus	Function
G	Glt-A	Glutamate synthesis
A	Aro-G	Isoenzyme mediating first step of aromatic pathway.
k) t) e) o)	gal operon	Galactose metabolism
λ ATT		Attachment site of prophage λ

FIGURE 6. Lengths of $F'8$, λ dg 17-8, λ dg T166 and deletion 9-3.

2) 3 X D Medium (Fraser and Jerrel , 1953) contains per litre, 10.5 g Na_2HPO_4, 4.5g KH_2PO_4, 1.0g NH_4Cl. To the above solution immediately before use was added per litre, sterile 1M $CaCl_2$, 0.3 ml; 15% Difco Bacto Casamino acids, 100 ml; 60% Glycerol, 50 ml; 10% $MgSO_4$, 5 ml. Appropriate supplements were also added (see below and table 6).

3) Minimal Medium contains per litre, 5g NH_4CL; 1g NH_4NO_3; 2g anhydrous Na_2SO_4; 3g K_2HPO_4; 1g KH_2PO_4; and 1.6g $MgSO_4$. The appropriate supplements were added immediately before use (see below).

4) Supplements. The supplements necessary for the growth and selection of various bacteria both in liquid and solid media were sterilised separately in appropriate amounts. These are summarised in table 6 together with the abbreviations used in the text.

5) NUTRIENT BROTH AGAR, contains 50g/1 Oxoid Nutrient Broth No.2 and 15g/1 Difco Bacto Agar.

6) MINIMAL AGAR. Minimal medium (see above) made to 1.5% agar with Difco Bacto Agar. Supplements were added immediately before pouring into plates (see above and table 6).

7) M.S. TOP AGAR contains per litre 10g Difco Bacto Tryptone, 8g NaCl, 1g Difco Bacto Yeast Extract and 8g Difco Bacto Agar.

All media with the exception of the medium used in the microferm were sterilised by autoclaving at a pressure of 15 lbs/sq.inch for 15 minutes. The medium in the microferm was autoclaved at 20 lbs/sq.inch pressure for 2 hours.

TABLE 6. Supplements to Basic Media

Supplement	Abbreviation	Final Concentration
Galactose	Gal.	0.2% (w/v)
Galactose (Purified, Sigma)	G.Gal.	0.2% (w/v)
Glucose	GLU	0.2% (w/v)
Histidine	H	0.02% (w/v)
Tryptophan	T	0.02% (w/v)
	5 x T	0.1% (w/v)
Streptomycin	Sm.	200 μg/ml

C. Buffers and Solutions

a) λ-dil (Kaiser and Hogness, 1960) contains O.O1M Potassium Phosphate pH 7.0, O.O1M $MgSO_4$, 10μg/ml Bovine Serum Albumin (Sigma).

b) Column Buffer contains O.3M NaCl; O.O1M NaAc pH 7.0.

c) Neutralised Column Buffer (N.C.B.) is column buffer neutralised with 3M Tris to pH 7.5.

d) Standard Saline Citrate buffer (SSC) contains O.15M NaCl, O.O15M Tri-sodium citrate. After mixing, the pH was adjusted to 7.5 with concentrated HCl.

e) RNAse is ribonuclease-A from Bovine pancreas, 5 x crystallized (Sigma). This was made up as O.1 mg/ml in 2 x SSC, O.O1M Tris pH 7.5 at $4^{o}C$ and boiled for 5 minutes to inactivate any DNAse in the preparation.

f) DNAse is Deoxyribonuclease-1 from Bovine pancreas, electrophoretically purified (Sigma). This was dissolved in O.1M potassium acetate pH 5.O to a final concentration of 2 mg/ml.

g) Sodium Lauryl Sulphate (SLS) was used as a 10% solution.

h) Polyethylene Glycol (P.E.G.) with a molecular weight of 2O,OOO and 6,OOO were both tried as a 30% solution. M.W. = 6,OOO was found to be the more convenient.

i) Dextran Sulphate (D.S.) M.W. = 2,OOO (Pharmacia) was used as a 20% solution.

j) I.C.R. Compounds were obtained from Dr. H.J. Creech, Institute of Cancer Research, Philadelphia. These acridine-like compounds were used as 1mg/ml solutions. These solutions were stored at -4°C in the dark.

k) Counting fluid contains 12.5 g POP, 750 mg POPOP in 2½ l. toluene and is dispensed in 15 ml amounts.

Unless otherwise stated the chemicals were obtained from British Drug Houses Ltd. Analar reagents were used whenever commercially available.

D. λ^+ Assay

100-fold serial dilutions of the phage suspension were made in M.S. Broth or pH 7.0 Buffer. 0.1 ml of each of these was mixed with 0.1 ml of an overnight culture of E.coli A24 ($\sim 10^9$ cells/ml) and incubated for 15 minutes at 37°C to allow adsorption. To this was added 2.0 ml of molten MS top agar equilibrated at 45°C in a water bath. The mixture was poured on to a nutrient broth agar plate. The plates were dried at room temperature, incubated at 37°C and scored the following day.

E. λdg Transduction Assay

10-fold serial dilutions of the phage suspensions were made in pH 7.0 buffer. 0.1 ml of appropriate dilutions was mixed with 0.2 ml of log phase bacteria, 9-3h$^+$ and 0.1ml λ^+ helper at a multiplicity of infection (moi) of $\sim$ 3.

3. The mixture was incubated at 35°C for 20 minutes. 0.1 ml of each mixture was then pipetted on to Gal 5 x T Sm plates and spread with a sterile glass spreader. The plates were incubated at 30°C for 3 days.

F. Isolation of Revertants

0.1 ml and 0.2 ml of overnight cultures were spread with a sterile glass spreader on to Gal 5 x H Sm plates. After 2 days incubation at 37°C colonies were picked and streaked on to G.Gal plates for testing.

G. Analysis of Revertants

Each possible revertant was grown up overnight and tested as follows.

i) F^1-duction

Aliquots of each revertant were spotted on streaks of E.coli A24 on Gal plates as a control for ability to mate and on 9-3h$^+$ on Gal 5 x T Sm plates. The results were interpreted as in table 7A.

ii) 0.1 ml of each revertant was mixed with 2.0ml of M.S. top agar equilibriated to 45°C, and plated on N.B. agar plates. These were allowed to set, then the following phages were spotted on to each plate:-

T_4^+, Am17, Am22, Am272 and f2. The spots were allowed to dry at room temperature then the plates were incubated overnight at 37°C and scored the following day.

TABLE 7. Interpretation of Revertant Analysis

A. F^1Duction

A24	9-3h$^+$	Type of Reversion
+	+	Back mutation of F^1Gal
+	-	Mutation on Bacterial Genome affecting Gal$^-$ on F^1 Gal
-	-	Loss of F^1 Gal.

B. Suppression

Am 17	Am 22	Am 272	T_4^+	f2	Type of Reversion
-	-	-	+	+	Back Mutation of F^1Gal.
+	+	+	+	+	Amber Suppressor
-	+	-	+	+	Ochre Suppressor
+	+	+	+	-	F^- Amber Suppressor
-	+	-	+	-	F^- Ochre Suppressor

An amber suppressor and an ochre suppressor in the same genetic background as the gal$^-$ bacteria and an su$^-$ female were used as controls.

The results were interpreted as in table 7B.

H. Reversions

Cultures of the gal$^-$ mutant bacteria were grown up overnight in M.S. Broth in a N.B.C. Gyrotory Shaker Air Bath at 37^{o}C. Serial tenfold dilutions were made in M.S. Broth.

0.1 ml of undiluted and 10^{-1} dilutions were spread on Gal 5H Sm plates, incubated for 3 days at 37^{o}C then scored. The viable colonies found were presumed to be revertants.

0.1 ml of appropriate dilutions of the overnight cultures were spread on N.B. plates and incubated overnight at 37^{o}C. This gave the total number of viable colonies in the culture.

I. Mutagenesis

The mutagens used were 2-Aminopurine, Nitrosoguanidine and the ICR compounds 191, 370 and 372.

Cultures of the gal$^-$ mutant bacteria were grown in M.S. Broth at 37^{o}C with shaking. 0.1 ml and 0.2 ml respectively of the cultures was spread on to Gal 5H Sm plates. A small crystal of N.G., a few grains of 2-A.P. or 5 λ of 1 mg/ml solution of the ICR compounds was placed ~~in the~~

in the centre of each plate. The experiment was carried out in duplicate and the plates incubated in darkness for 3 days.

J. Making of λ-Lysogens and λ Resistant Stocks

0.1 ml of an overnight culture of the bacteria grown in M.S. Broth was mixed with 2.0 ml of M.S. Top agar equilibriated at 45°C and spread on a N.B. agar plate. λ^+ was spotted on to these. After allowing the spots to dry the plates were incubated overnight at 37°C. A loopful from the centre of the area of lysis was picked and streaked out on to a minimal agar plate containing the appropriate supplements and incubated for 2 days at 37°C.

Colonies were picked from these and spot tested with λ^+, λ^V, T_4^+ and R_{II} 1272. Incubation was again overnight at 37°C and the plates were scored as shown in Table 8.

λ resistant bacteria were made by the same method using λ^V instead of λ^+ as the initial spot.

K. Cold λ^+ and λ dg Preparations for DNA

Nitrogen Mustard induction of a λ lysogen was used initially. When a well controlled and rapid method for changing the temperature of the culture had been developed, however, heat induction of a temperature sensitive λ lysogen was adopted. λ^+ and λ dg titres were followed by assaying each fraction throughout the procedure.

TABLE 8. Bacterial Response to Bacteriophages

λ^+	λ^v	$r_{II}1272$	T_4^+	Interpretation
+	+	+	+	λ Sensitive
-	+	-	+	λ Lysogenic
-	-	+	+	λ Resistant

b) Heat Induction

i) A 500 ml culture of a temperature sensitive λ lysogen was grown overnight at 33°C. This was initially grown in M.S. Broth but later minimal medium plus galactose was used to prevent the segregation of the λ dg. This was innoculated into 11 litres of prewarmed M.S. Broth in the Microferm Fermentor, giving an initial OD_{650} reading of approximately 0.1-0.2 units.

ii) The culture was grown at 33°C until OD_{650} = 0.6-0.7.

iii) The thermostat was then adjusted to allow a temperature of 42°C and 2 litres of fresh M.S. Broth at 75°C was added. Care was taken to stir in the hot medium as vigorously as possible. It took approximately 5 minutes to attain 42°C.

iv) After 15 minutes at the 42°C setting, the thermostat was adjusted to give a temperature of 37°C, and the culture incubated until lysis occurred. It again took approximately 5 minutes for the new temperature to be reached.

v) At lysis 50 mls of chloroform was added and the culture stirred and aerated for a further 30 minutes.

L. Purification of λ^+ and λ dg Preparations

Initially the ammonium sulphate precipitation method was used. Since this gave a very low yield - approximately 10% of the initial titre - a two phase system was later adopted.

(a) Ammonium Sulphate Precipitation. (Kaiser and Hogness, 1960).

i) The entire culture was centrifuged through the Servall continuous flow apparatus at $0^{o}C$ and maximum speed and at a rate of 100 ml per minute. The supernatant was collected in a chilled aspirator.

ii) 250g/l ammonium sulphate was added with stirring at $4^{o}C$.

iii) This was left overnight then centrifuged at $0^{o}C$ through the continuous flow apparatus of the Servall at maximum speed and a rate of 40 ml/minute.

iv) The pellet was resuspended in 300 ml of λ -dil and dialysed for 24 hours at $4^{o}C$ against 3 changes of 4 l. of 0.01 M $MgSO_4$, 0.01M Potassium Phosphate pH 7.0 buffer.

v) It was then centrifuged for 10 minutes at 14,000 g. The pellet was extracted two more times with 25 ml of Mg-Phosphate buffer.

vi) The combined supernatants were centrifuged at 21,000 g. for 3 hours in a Spinco model L centrifuge and the pellet taken up in a final volume of 120 ml (40% of the Ammonium sulphate precipitation fraction).

vii) This was dialysed against 3 changes of 4 litres each of 0.01M $MgSO_4$, 0.01 M Potassium phosphate pH 7.0 for 24 hours.

(b) 2-Phase system. (Philipson et al., 1966).

The bacterial debris was initially removed by centrifugation through the Servall continuous flow apparatus but the following method was later adopted. All steps were carried out in the cold.

i) 25g/1 NaCl was added to the culture and stirred until dissolved.

ii) 93.3 ml/1 30% PEG and 10 ml/litre original volume D.S. were added and the mixture stirred for 1 hour then left to stand overnight.

iii) The lower phase containing the bacterial debris was run off and discarded.

iv) 300 ml/litre of PEG and O.O1 vol. of D.S. were added with stirring when debris was removed by centrifuging and 207 ml/1 PEG and 7.12 ml D.S. when the debris was removed by the 2-phase method. In both cases the mixture was stirred for 1 hour after addition and the procedure in both cases continued as follows.

v) The mixture was left to stand for 21/2-3 days.

vi) The lower phase and interphase were run off and the remainder left overnight.

vii) The remaining lower phase and interphase were again collected and pooled with the previous collection.

viii) The pooled suspensions were centrifuged in the G.S.A. rotor of the Servall at 8,OOO rpm for 20 minutes and as much upper and lower phase removed as possible.

ix) The interphase and remaining upper and lower phases were centrifuged for 20 minutes at 12,OOO rpm and all the upper phase plus as much as possible of the lower phase removed.

x) The interphase was taken up in 0.02M Tris pH 7.5 at $0^{o}C$ and 0.15 volume 3 M KCl was added to precipitate the D.S.

xi) This was left overnight, then spun at 8,000 rpm in the G.S.A. rotor of the Servall for 1 hour.

The supernatant was removed by pipette and stored. The pellet was resuspended in 25 ml 0.02 M Tris pH 7.5 at $0^{o}C$, 0.5 M KCl and step xi) repeated two more times.

All fractions were assayed for plaque forming units and also for transducing titres when λ dg was being prepared.

(c) Caesium Chloride Density Centrifugation

i) CsCl was added to the partially purified phages to make the final suspension 41% CsCl (w/w) having a density at $4^{o}C$ of $1.46_{g}/cm^{-3}$.

ii) This was centrifuged for 1 hour at 21,000 g and the clear liquid between the pellet and pellicle transferred to a fresh tube.

iii) This was centrifuged at $17^{o}C$ for 42 hours at 40,000 rpm in the 40,000 rotor of the Spinco model L centrifuge. Two distinct bands of λ^{+} and λ dg phages wereseen when λ dg preparations were made.

iv) The gradient was fractionated by piercing the bottom of the tube with a needle. 5 drop fractions were collected. To each fraction was added 1.0 ml, 0.02 M Tris pH 7.5 at $0^{o}C$, 0.5 M KCl and this was left overnight

before mixing. The optical density of the diluted fractions was read at 260μ. Figure 7A shows a typical OD_{260} profile of phages prepared from T166/λ bacteria. Figure 7B (kindly supplied by Mr. J. Gosden) shows the profile of phages from 17-8/λ bacteria.

v) The peak fractions were collected and dialysed against three changes of 0.1 M NaCl, 0.01 M Tris pH 7.5 at $0^{o}C$.

vi) Storage was at $4^{o}C$ over chloroform.

M. P^{32}-labelled λ^{+} - Preparation and Purification

i) An overnight culture of E1(857)/λ in M.S. Broth was innoculated into 100 ml M.S. Broth to an OD_{650} of approximately 0.1 units.

ii) This was grown in the N.B.C. Gyrotory shaker air bath at $32^{o}C$ to an OD_{650} = 0.2. 2 mc of P^{32} as orthophosphate was added.

iii) The optical density was followed until 0.6 units then the culture transferred to a waterbath at $42^{o}C$ and kept there for 15 minutes with aeration.

iv) The culture was replaced in the shaker and grown at 37^{o} to lysis.

v) Chloroform was added and the shaking continued for a further 30 minutes. The culture could be left overnight at $4^{o}C$ if necessary.

vi) The culture was removed from the chloroform and spun at 10,000 rpm for 10 minutes to remove debris.

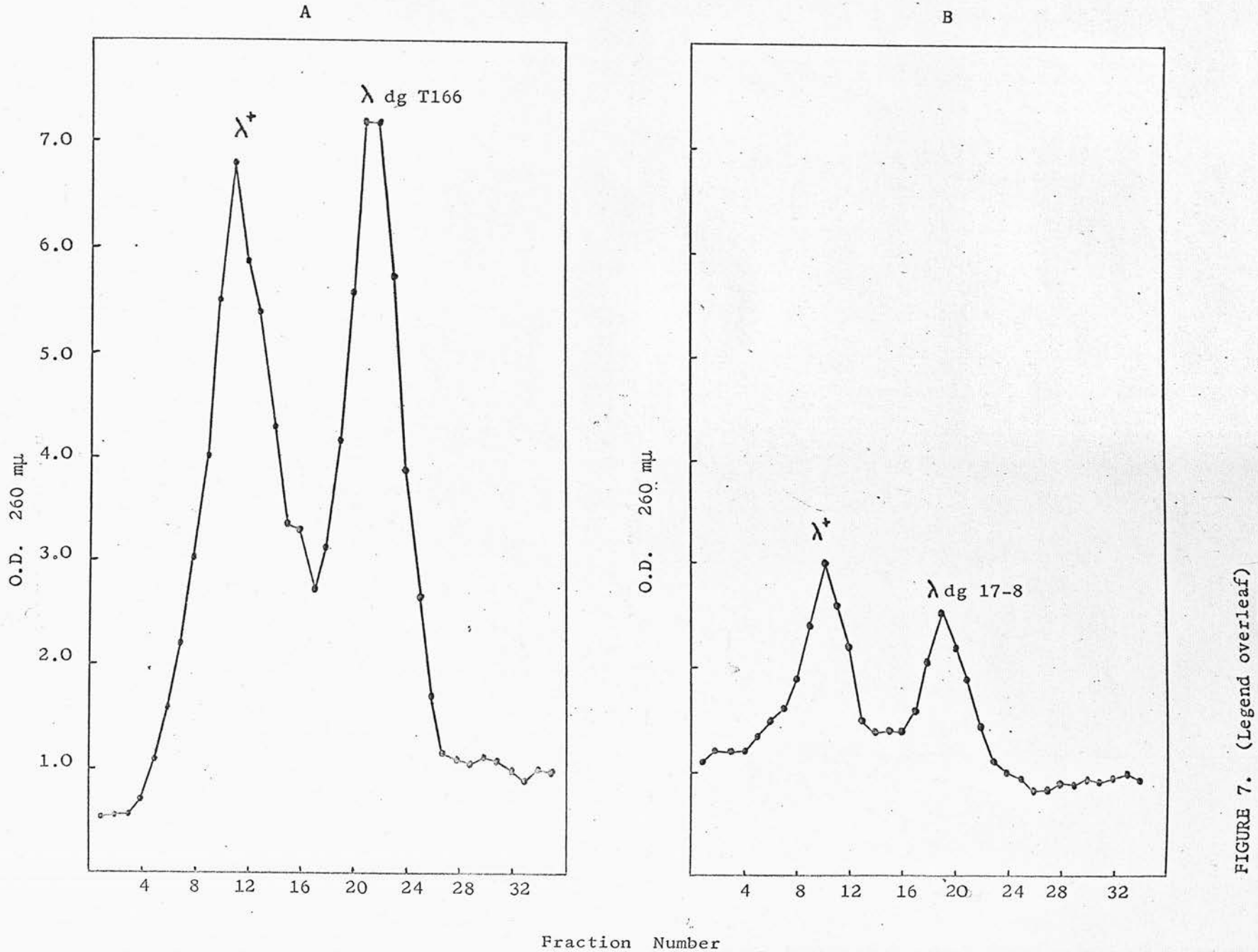


FIGURE 7. (Legend overleaf)

LEGEND TO FIGURE 7

Optical Density Profiles of Caesium Chloride Gradients of Phages Prepared from T166/λ and 17-8/λ Bacteria

CsCl density = $1.46_9 / cm^{-3}$ at $4^{o}C$; Centrifugation was for 1 hour at 21,000 g then the clear liquid separated from pellet and pellicle and run 42 hours at 40,000 rpm in the Spinco model L centrifuge at $17^{o}C$.

Fractionation was from the bottom of the tube via an inserted needle. Fraction size was 5 drops and this was diluted with buffer, as explained in the text, before the optical density at 260 mμ was read.

A. Phage preparation from T166/λ bacteria

B. Phage preparation from 17-8/λ bacteria

vii) The supernatant was centrifuged for 90 minutes at 30,000 rpm and 0°C, and the pellet resuspended in 0.1 M Tris, 0.01 M NaCl.

viii) CsCl was added to make the suspension 41.5% CsCl i.e. ρ_4 = 1.46.

ix) This was run at 45,000 rpm for 20 hours at 17°C.

x) The phage band was removed with a pasteur pipette and dialysed overnight against 3 changes of 0.1M NaCl, 0.01 M Tris pH 7.5 at 4°C.

xi) Storage was at 4°C over chloroform.

N. P^{32} Labelled λ^+ and λ dg DNA Preparations

i) A suitable amount of P^{32} labelled phage was diluted with either cold λ^+ or λ dg and 0.1 M NaCl - 0.01 M Tris to give the required phage concentration and specific activity.

ii) To this was added an equal volume of redistilled phenol and the complete mixture gently mixed for 5 minutes at room temperature.

iii) This was then centrifuged at 10,000 rpm for 10 minutes at room temperature.

iv) The phenol layer was removed.

v) Two more phenol extractions were carried out and in the latter case the aqueous phase was removed with a wide mouthed pipette and dialysed against 1/10 S.S.C. at 4° to remove the phenol. The dialysis tubing used had been previously treated with zinc chloride as follows.

This increases the pore size of the tubing.

The dialysis tubing was soaked in 64% (w/v) $ZnCl_2$ for 15 minutes at room temperature. It was then washed 3 times with distilled water followed by 1 wash in O.1 N HCl and a further 3 washes in distilled water. It was then brought to the boil in O.O1M EDTA and transferred to distilled water. Long term storage was in 50% glycerol at $4^{o}C$.

O. Growth of Bacteria for RNA Preparation

(a) Unlabelled.

i) An overnight culture grown in 3XD medium was innoculated into 400 ml 3 XD to an OD_{650} of less than 0.1. The appropriate supplements had been added to both media.

ii) The culture was grown in a 37°C water bath with good aeration and the OD_{650} followed.

iii) At OD_{650}= between 0.2 and 0.3 the culture was either induced by the addition of 4.0 ml of 0.5 M Fucose (Final concentration = 5.0×10^{-3}M) or repressed by the addition of 10 ml of 0.1 M TMG (final concentration 2.5×10^{-3}M).

iv) Growth was continued until OD_{650} = 0.8 was reached, then the culture was poured over 160 mls crushed buffer ice made at -40°C. The buffer contained 0.01 M NaCl, 0.01 M Tris (pH 7.5 at 0°C) and 0.01 M NaN_3.

v) The mixture was stirred until the temperature dropped below 5°C. This took approximately 30 secs to attain.

vi) The cells were pelletted in the GSA rotor of a Servall centrifuge at maximum speed.

vii) The pellet was resuspended in 5 mls 0.01 M NaN_3, 0.005 M EDTA, 0.02 M NaAc, 0.1 M NaCl, and could be stored overnight at -20°C if required.

(b) Radio-actively labelled.

The method was the same as above and the label was added as follows.

While the culture was growing the generation time was calculated. At OD_{650} = 0.8, 0.1 mc of C^{14} Uracil was added and the mixture shaken by hand ~~at room temperature~~ for 1% of the generation time. The mixture was then poured over crushed buffer ice as described above.

P. RNA Preparation

i) The cell suspension was thawed if necessary and mixed well to disaggregate the cells.

ii) 0.25 volume of 10% S.L.S. was added and the mixture incubated at 45°C for 2 minutes to lyse the cells.

iii) An equal volume of redistilled water saturated phenol at 50°C was added. The mixture was shaken at 50°C for 5 minutes then at room temperature for 5 minutes.

iv) The mixture was centrifuged at room temperature for 10 minutes at 10,000 rpm. The phenol layer was removed and discarded.

v) An equal volume of phenol was added and the mixture shaken at room temperature for 10 minutes. This was then centrifuged for 10 minutes at 10,000 rpm.

vi) The aqueous phase was removed by pipette. An equal volume of buffer was added to the phenol and the mixture shaken and centrifuged as in step v).

vii) The phenol layer was removed and replaced with fresh phenol. The mixture was shaken and centrifuged as above.

viii) The final aqueous layer was removed and pooled with the previous one.

ix) An equal volume of absolute alcohol was added to the pooled aqueous layers and the DNA spooled out with a glass rod. This was dissolved in 4 ml 0.05 M Tris pH 7.5 at room temperature, 0.005 M $MgCl_2$.

x) A second volume of Absolute alcohol was added to the pooled aqueous layers. This was left standing for at least 15 minutes then centrifuged at 10,000 rpm for 10 minutes at $4^{o}C$.

xi) The pellet was taken up in 2 ml 0.05 M Tris - 0.005 M $MgCl_2$.

xii) Both solutions of alcohol precipitated material were treated with DNAse at a final concentration of 50 μg/ml for 15 minutes at $37^{o}C$.

xiii) The two solutions were mixed and 6 mls of water saturated redistilled phenol was added. The mixture was shaken at room temperature and centrifuged as before. The phenol layer was discarded.

xiv) 6 mls of phenol at $50^{o}C$ was added to the aqueous layer. This was shaken for 5 minutes at $50^{o}C$ then 5 minutes at room temperature. It was then centrifuged.

xv) The aqueous phase was removed and the residual phenol removed from it by 3 ether extractions each with 4 volumes of ether.

xvi) Air was then bubbled through to remove the ether.

xvii) O.1 volume of 3 M NaCl, O.1 M NaAc pH 5.O was added. The resulting solution, now in column buffer was passed through a 40 cm x 3cm SE 5O Sephadex column and eluted with column buffer.

xviii) Fractions containing more than 5OO μg/ml RNA were collected. The OD_{260}/OD_{280} ratio was 2.O.

xix) The pooled RNA fractions were concentrated when necessary by precipitation with 2 volumes of absolute alcohol. The precipitate was pelleted by centrifugation and taken up in an appropriate volume of N.C.B.

When concentration was not necessary the pooled fractions were neutralised to pH 7.5 with 3 M Tris.

xx) The RNA was stored at -15°C.

Q. Hybridisation

This was carried out according to the method of Gillespie and Spiegelman (1965) in which the DNA is denatured and fixed on nitrocellulose membranes.

(a) Denaturation of DNA and Loading of Membranes.

i) P^{32} DNA prepared as previously described was diluted by an appropriate amount with 1/1O SSC.

ii) The DNA was denatured by alkali. The pH was raised to 12.0 with 1 M NaOH and left for 10 minutes at room temperature. The solution was then neutralised to pH 7.5 with 0.4 N HCl, and immediately transferred to an ice bath.

iii) It was adjusted to a suitable concentration with distilled H_2O and made 6 x SSC with an appropriate amount of 20 x SSC.

iv) Nitrocellulose membranes were soaked in 2 x SSC for 5 minutes then placed on the filtration apparatus under suction. They were washed once with 35 ml of 2 x SSC under suction.

v) 1.0 ml of the denatured DNA solution was loaded under gravity on to each membrane. The amount of DNA loaded per membrane is stated in the text.

vi) The membranes were then washed with 2 volumes, each of 35 ml of 2 x SSC under suction.

vii) Blanks were loaded in the same manner except that 6 x SSC solution was used instead of the denatured DNA solution.

viii) The loaded membranes were then treated in one of the following ways.

1) Single filters were incubated separately at room temperature for a minimum of 4 hours then at 80°C under vacuum for a further 2 hours.

2) The membranes were rolled up and placed in small test tubes either singly or in groups of three. The groups comprised either 1 Blank, 1 λdg DNA and 1 λ^+ DNA membranes or 1 Blank and 2 λ dg DNA membranes. The tubes were placed in a dessicator at 4°C for a minimum of 2 hours then in a vacuum oven at 80°C for 2 hours. The method used is stated in the text.

(b) Annealing and Disaggregation.

i) Single filters were annealed in 25 ml beakers, those put into tubes after loading were annealed in the tubes. Incubation was at 65°C in a liquid paraffin bath in the standard hybridization mix containing 100 µg/ml cold rat liver RNA in N.C.B. (to reduce non-specific binding), E.coli RNA in N.C.B., O.3 M NaCl, O.O3 M tri-sodium citrate and N.C.B. to make up to volume. When annealing was in beakers a layer of paraffin wax was added and tubes were stoppered to prevent evaporation. For saturation experiments different amounts of C^{14} labelled RNA was added. For competition experiments a fixed amount of C^{14} RNA and various amounts of unlabelled RNA was added.

ii) After incubation 20 mls of 2 x SSC prewarmed to 65°C was added to the beakers, or the filters incubated in tubes were removed, separated, and placed in 20 ml of prewarmed 2 x SSC in 25 ml beakers. In both cases the membranes were incubated for a further hour to allow disaggregation of unbound RNA from the membranes. The beakers were then chilled in ice. At this point the paraffin wax was removed from those beakers which contained it during annealing.

iii) All filters were then washed under suction with 2 x SSC. Both sides were washed with 5 volumes of 35 ml.

iv) The membranes were then treated with a final concentration of 20 µg/ml RNAse for 15 minutes at 37°C. The containers were again chilled.

v) Both sides of the membranes were again washed 5 times as before, and the washed membranes placed in new counting vials. They were dried under vacuum and cooled to room temperature before 15 ml of Toluene counting fluid was added.

vi) The counts per minute on the membranes were counted in a Packard Scintillation counter. The average of three 10 minute counts was taken.

(c) Annealing time.

To establish the time of incubation necessary to reach equilibrium in our hybridization system, time course experiments were carried out.

A constant amount of labelled E1/F8 induced RNA was hybridized to λ dg DNA for several different lengths of time.

Figure 8 shows the counts per minute RNA hybridized per μg DNA plotted against the time of incubation. Experimental details are given in the legend to the figure.

By 40 hours of incubation the reaction is over and this time period was chosen for the majority of the experiments to be described. When a different incubation period was used this is given in the legend to the figures illustrating the results obtained.

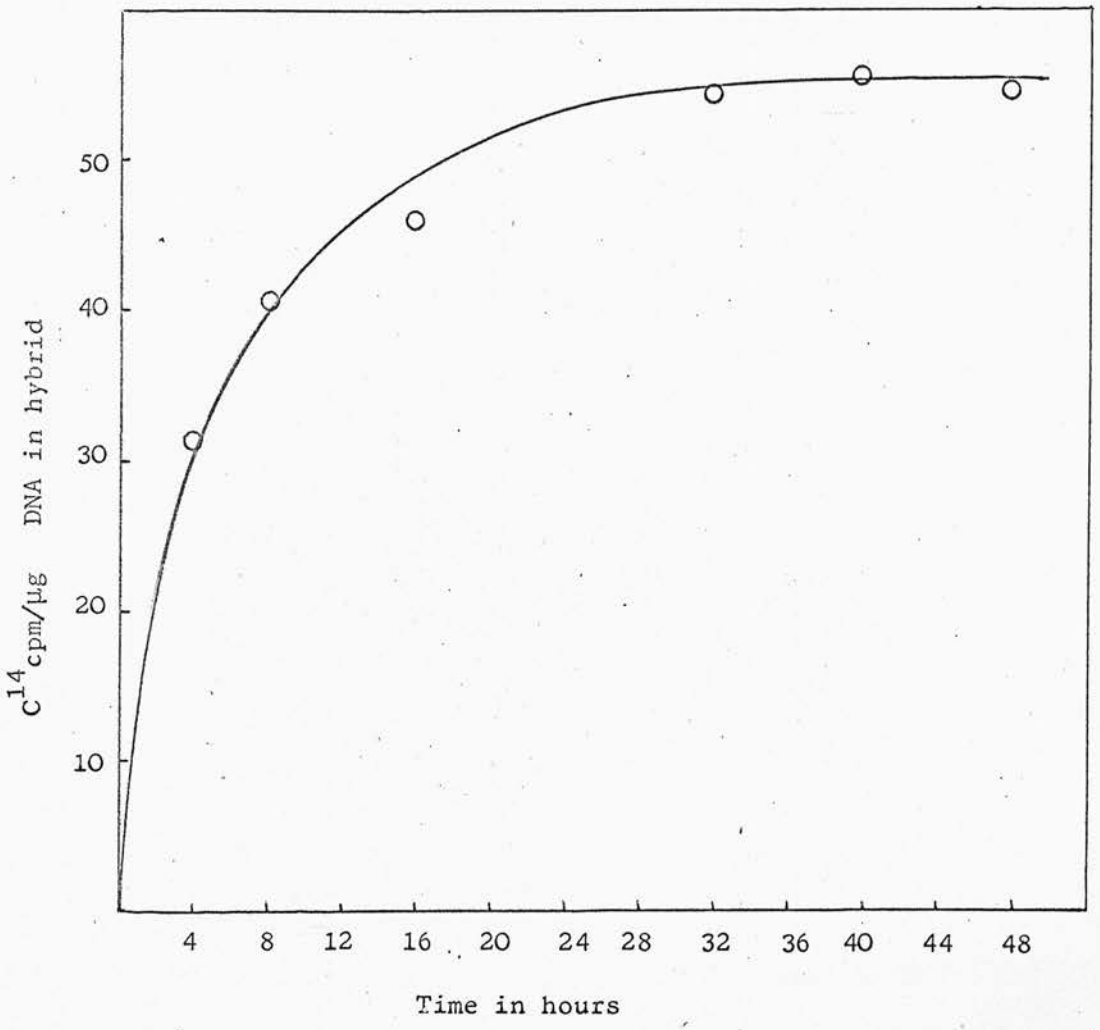


FIGURE 8. Time Course Experiment with λdg 17-8 DNA and E1/F8 Induced RNA

The membranes were loaded with 2.5 µg λdg 17-8 DNA; hybridization was carried out in test tubes each containing 2 λdg and 1 Blank membrane in 0.6 ml of standard hybridization mix (100 µg/ml rat liver RNA, 0.30 M NaCl, 0.03 M tri-sodium citrate, E.coli RNA). The amount of E.coli RNA added to each tube was constant (434 µg). The tubes were incubated for 4,8,16,24,32,40 and 48 hours respectively.

III. ANALYSIS OF RESULTS

I. Symbols

r = RNA hybridized per μg DNA in μg.

r_s = RNA hybridized per μg DNA at infinite RNA concentration in μg.

h = Concentration of labelled RNA.

H = Ratio of total labelled RNA to DNA.

g = Concentration of unlabelled RNA.

G = Ratio of total unlabelled RNA to DNA.

D = Amount of DNA present in hybridization vessel in μg.

p = Proportion of total hybridizible DNA sequence to which complementary sequences are present in both labelled and unlabelled RNA preparations.

q = Proportion of total hybridizible DNA sequence to which complementary sequences are present in labelled but not unlabelled RNA preparations.

$p + q$ = 1.0

F = The apparent concentration of common sequences in labelled and unlabelled RNA.

F_G = The apparent concentration of Galactose mRNA in labelled and unlabelled RNA.

c = Radioactivity (cpm/μg DNA) in the RNA-DNA hybrid.

Cp = Proportional competition = $\frac{c_o - c}{c_o}$ where c_o and c are the radioactivities in the RNA-DNA hybrid in the absence and presence of cold competitor respectively.

s.a. = Specific activity of labelled RNA which specifically hybridizes to the DNA.

$s.a._G$ = Specific activity of gal mRNA.

α = Proportion of total RNA which is gal mRNA.

m = N^o of gal mRNA molecules per cell.

K, K_1, K^1 etc. = constants

II. Saturation Data

In hybridization experiments using a fixed amount of T_4 DNA and increasing amounts of T_4 RNA made 'in vitro', Bishop et al (1969) showed that a linear relationship exists between the reciprocals of the amount of RNA in the DNA-RNA hybrid and the input RNA concentration. The equation of this line was found to be $1/r = K.1/h + K_1$.

Assuming that a linear extrapolation to $K/h = O$ is valid, then $K_1 = 1/r$ at infinite RNA concentration, i.e. $K_1 = 1/r_s$.

Thus $$1/r = K/h + 1/r_s \quad \ldots\ldots\ldots\ldots \quad (1)$$

This equation can be rearranged:

$$h/r = K + h/r_s \quad \ldots\ldots\ldots\ldots \quad (2)$$

which represents the straight line plot $^{h}/_{r}$ versus h. In this form a more accurate value for r_s can be obtained from the slope of the line, and a standard error can be attached to it. Similarly a value for K can be obtained from the slope of the line of equation (1).

Throughout the T_4 experiments RNA was in excess and the time of incubation (16 hrs) was sub-optimal with respect to maximal hybridization of RNA to DNA. Thus the RNA concentration after the incubation period was not greatly changed from the initial concentration. The amount of hybrid formed per μg DNA is therefore dependent only on the input concentration of RNA and is independent of DNA concentration.

The conditions of the experiments to be described here, differ from those outlined above. The RNA used was 'in vivo' RNA and thus the specific hybridizible fraction was a relatively small proportion of this. The λ DNA molecule is four times smaller than T_4 and thus the gene concentration per μg DNA is four times higher in this case. A greater amount of DNA and longer incubation time is used here so that the hybridization reaction proceeds further towards completion. The amount of hybrid formed per μg DNA in this case was found to be dependent on the ratio of input RNA to DNA.

To obtain saturation curves we plot r against H (where $H = {}^{h}/_{D}$). Thus the reciprocal plots become $^{1}/_{r}$ versus $^{1}/_{H}$ giving the equation for the straight line:-

$$1/r = K/H + 1/r_s \quad \text{.....} \quad (3)$$

and H/r against H, giving the equation

$$H/r = H/r_s + K \quad \text{.....} \quad (4)$$

In practice, however, the amount of RNA hybridized (r) cannot be found with 'in vivo' RNA where the specific activity of the hybridizible RNA fraction is not known before the data are analysed. We therefore plot the radioactivity of the RNA in the RNA-DNA hybrid (cpm/μg DNA = c) against H to obtain saturation curves.

Since $c = r \times s.a.$ Equation (3) becomes

$$1/r \times s.a. = K/s.a. \; 1/H + 1/r_s \times s.a.$$

$$\text{i.e. } 1/c = K^1/H + 1/c_s \quad \text{.....} \quad (5)$$

and Equation (4) is converted to

$$H/r \times s.a. = H/r_s \times s.a. + K/s.a.$$

$$\text{i.e. } H/c = H/c_s + K^1 \quad \text{.....} \quad (6)$$

Where $K^1 = K/s.a.$ in both cases.

Since the DNA retained on membranes varies, we used ^{32}P labelled DNA in order to measure accurately the amount on each membrane. In this way a direct comparison can be made between the c_s values obtained from experiments using different amounts of DNA/membrane and experiments in which different numbers of filters were used per hybridization vessel.

The E.coli DNA carried on λ dg 17-8 comprises the complete gal operon plus the adjacent regions of the E.coli genome (see figure 6) which we term Non-gal. Since RNA from E.coli will hybridize to both the Gal and Non-Gal DNA it is necessary to divide the cpm/μg DNA in the hybrid into these two components

i.e. $c = c_G + c_{NG}$ where c = Total cpm/μg DNA in hybrid

c_G = cpm/μg DNA in hybrid due to Gal mRNA

c_{NG} = cpm/μg DNA in hybrid due to Non-gal mRNA.

In order to do this we used λ dg T166, which carries the Non-gal region between λ^{att} and the gal operon and the epimerase gene, only, of the gal region (see figure 6). By comparison of the amount of hybridization of RNA to DNA from λ dg 17-8 and λ dg T166 we obtain a set of estimates of the cpm/μg DNA in the hybrid due to RNA homologous to the K + T region of the DNA (See Results section IIc).

i.e. $c_{(17-8)} - c_{(T166)} = c_{(K+T)}$

where $c_{(17-8)}$ = cpm/μg DNA in hybrid using DNA from λ dg 17-8

$c_{(T166)}$ = cpm/μg DNA in hybrid using DNA from λ dg T166

$c_{(K+T)}$ = cpm/μg DNA in hybrid due to hybridization of RNA homologous to K+T region.

The molecular weights of the polypeptides specified by the three cistrons of the gal operon have been estimated. Wilson and Hogness (1966) showed kinase to consist of one polypeptide of molecular weight 4×10^4 and epimerase to comprise two identical subunits plus a closely bound molecule of DPN with a total molecular weight of 7.9×10^4 (Wilson and Hogness, 1964, 1966). Saito et al.(1967) showed that the transferase molecule was made up of two identical subunits, each of molecular weight 40,000.

Thus the basic polypeptide unit in all three enzymes has a molecular weight of approximately 4×10^4. We can conclude from this that the genes coding for these polypeptides are of roughly equal size.

As added evidence Guha et al.(1968) have shown that roughly equal amounts of induced E.coli gal^+ RNA hybridizes to each of the three gal genes. They did this by successively hybridizing the RNA with λ dg's carrying the e cistron, e + t cistrons and finally the complete gal operon.

Thus $(K + T + E) = {}^{3}/_{2} \cdot (K + T)$

and $c_G = {}^{3}/_{2} \cdot {}^{c}(K + T)$

This is the best estimate of c_G available, although we recognise that the E.coli DNA on λ dg 17-8 carried at the distal end of the gal operon is being ignored.

By plotting a series of c_G values against H, a saturation curve is obtained and by plotting ${}^{1}/_{c}$ against ${}^{1}/_{H}$, and ${}^{H}/_{c}$ against H values of K_G^1 and c_{SG} respectively are found. By substitution of the c_G values into the

equation $c = c_G + c_{NG}$ a series of values of c_{NG} are obtained. These can then be used to find c_{SNG} and K^1_{NG} in the same way as above.

Using this derived gal data we can further estimate the specific activity of the gal mRNA ($s.a._G$), the proportion of the total RNA which is gal mRNA (α) and the number of molecules of gal mRNA per cell (m) as follows.

Let X = Proportion of total DNA which hybridizes with gal mRNA.

$$\therefore X = \frac{M.W.\,Gal}{M.W.\,\lambda dg} \times \text{Hybridization Factor}$$

Estimate of M.W. Gal = $6 \times 3 \times 40{,}000 = 7.2 \times 10^5$ daltons

M.W. $\lambda dg \approx$ M.W. $\lambda^+ = 3.0 \times 10^7$ daltons

Hybridization Factor = 0.6 (i.e. maximum amount of hybridization in our system).

$\therefore X = 0.014$

Also X = µg gal mRNA which hybridizes per µg DNA.

$$\therefore s.a. = {}^{c_{SG}}/_{X} = {}^{c_{SG}}/_{0.014}$$

Let K_G = value of ${}^{H}/_{c}$ when H approaches 0.

Then, assuming

$1/K_G$ = Gal counts hybridizible per µg RNA Input

$$\alpha = \frac{1/K_G}{s.a.} = \frac{0.014}{K_G \cdot c_{SG}}$$

where α = μg gal mRNA hybridizible per μg RNA input

= Proportion of total RNA which is gal mRNA.

Let R = gms of RNA/cell = 1.25×10^{-12} (Lavallé and De Hauwer, 1968).

$\therefore$ Gal mRNA per cell = $\frac{R.0.014}{K_G \cdot c_{SG}}$ gms.

$\therefore$ m = $\frac{\text{Av. R.0.014}}{\text{Gal M.W.} K_G . c_{SG}}$ where Av. = Avogadro's number

m = N^o gal mols/cell

= $\frac{\text{Av.}\alpha\text{.R}}{\text{Gal M.W.}}$

$\therefore$ $\underline{\underline{m = 1.04 \times 10^5 \times \alpha}}$

III. Competition Data

From Equation (5) we obtain

$$c_o = \frac{Hc_s}{K^1 c_s + H}$$ where c_o = cpm/μg DNA in hybrid in the absence of cold competitors

$$\text{Thus } c_o = (p + q).\frac{Hc_s}{K^1 c_s + H} \quad \ldots\ldots\ldots\ldots \quad (7)$$

Keeping the amount of labelled RNA constant and adding increasing amounts of unlabelled RNA does not alter the concentration or specific activity of the non-competing species of RNA which hybridize with the DNA. The effective concentration of competitor, however, now becomes $H + {}^{G}/_{F}$ and its specific activity $\frac{H.sa.}{H + {}^{G}/_{F}}$

Thus c_s becomes $\frac{c_s}{s.a.} \cdot \frac{H.\,sa}{H + {}^{G}/_{F}} = \frac{c_s H}{H + {}^{G}/_{F}}$

and $$c = \frac{p\,Hc_s}{K^1 c_s + H + {}^{G}/_{F}} + \frac{q\,Hc_s}{K^1 c_s + H} \quad \ldots. \quad (8)$$

where c = cpm/μg DNA in hybrid in the presence of cold competitor.

When homologous labelled and unlabelled RNA species are used it is obvious that $p = 1.0$ and $q = 0$.

i.e. $$c = \frac{Hc_s}{K^1 c_s + H + {}^{G}/_{F}} \quad \ldots\ldots\ldots\ldots\ldots \quad (9)$$

The labelled RNA used in all our experiments is prepared from induced cultures of E.coli wild type for the gal operon. E.coli E1/F8 and E1 are gal^+ (see Materials and Methods) and produce the gal enzymes to some extent under both repressed and induced conditions, thus unlabelled RNA prepared from these is homologous to the labelled preparation.

Likewise, the polar mutants under test produce low levels of kinase and transferase under the growth conditions used, showing that mRNA for these genes is present in the cells. Since we assume that gal mRNA is polycistronic it follows that epimerase mRNA must also be present. This being the case, the proportion of the DNA sequence for which complementary RNA is found in both hot and cold RNA is again 1.0 (i.e. p = 1.0 and q = 0). Hence Equation (9), in all our competition experiments can be solved for F, the only unknown.

As in the case of the saturation data we must divide the competition data into gal and non-gal components to allow evaluation of F_G.

Thus Equation (9) becomes

$$c_G = \frac{Hc_{SG}}{K_{NG}^{1} c_{SG} + H + {}^{G}/_{F_G}} \quad \ldots\ldots\ldots \quad (10)$$

for the cpm/μg DNA in the hybrid due to gal RNA.

and

$$c_{NG} = \frac{Hc_{SNG}}{K_{NG}^{1} c_{SNG} + H + {}^{G}/_{F_{NG}}} \quad \ldots\ldots\ldots \quad (11)$$

for the cpm/μg DNA in the hybrid due to non-gal RNA.

The concentration of the non-gal component in the total RNA presumably does not alter in the different mutants used or under induced or repressed

conditions. Thus F_{NG} = 1.0 in all cases and therefore values for c_{NG} can be calculated for each value of H. By subtracting this from the actual values of c found experimentally we obtain the corresponding values of c_G.

To calculate the values of the c_{SG} and c_{SNG} components of the total saturation value of the labelled RNA preparation used in each competition experiment the following formulae are applied.

$$c_{SNG2} = c_{SNG1} \times \frac{c_{S2}}{c_{S1}}$$

and $$c_{SG2} = c_{SG1} \times \frac{c_{S2}}{c_{S1}}$$

where c_{S1}, c_{SNG1} and c_{SG1} are the c_S values obtained as previously described from data of the saturation of λ dg 17-8 and λ dg T166 DNA's by E1/F8 induced RNA.

c_{S2} is the c_S value of the labelled RNA in the competition experiment.

c_{SNG2} and c_{SG2} are the gal and non-gal components of c_{S2}

Since $K_G^1 c_{SG}$ and $K_{NG}^1 c_{SNG}$ are constants, the values of these found from the λ dg T166 and λ dg 17-8 saturation experiments can be used in equations (10) and (11) respectively.

The sum of c_{SG2} and c_{SNG2} is found to ~~vary~~ differ slightly from the actual values of c_{S2} found. Thus a correction factor $\left(\frac{c_{S2}}{c_{SNG2} + c_{SG2}}\right)$ is introduced. Multiplication of the calculated values of c_{SG2} and c_{SNG2} by the correction factor gives the estimates of c_{SG2} and c_{SNG2} which are used for our analysis.

The proportional competition between gal mRNA in the two competing species of RNA's (C_{pG}) is found from the equation

$$C_{pG} = \frac{c_{OG} - c_G}{c_{OG}}$$

where c_{OG} = Gal cpm/μg DNA in the hybrid in the absence of competitor

$$= \frac{Hc_{SG}}{K_G^1 c_{SG} + H} \qquad \text{(from equation 5)}$$

c_G = Gal cpm/μg DNA in hybrid in presence of competitor

$$= \frac{Hc_{SG}}{K_G^1 c_{SG} + H + G/F_G} \qquad \ldots\ldots\ldots \qquad (10)$$

Thus $$G/C_{pG} = G/p + \frac{F_G(H + K_G^1 c_{SG})}{p} \quad \ldots \qquad (12)$$

which simplifies to

$$1/C_{pG} = 1/p + \frac{F_G(H + K_G^1 c_{SG})}{pG} \quad \ldots \qquad (13)$$

By plotting $1/C_{pG}$ against $1/G$ we obtain the straight line of equation (13) with the slope being equal to $\frac{F_G(H + K_G^1 c_{SG})}{p}$ and the intercept on the Y axis

equal to $1/p$. Since we take p = 1.0 the slope of the line equals $F_G(H + K_G^1 c_{SG})$ when the line passes through 1.0 on the Y axis.

When homologous labelled and unlabelled RNA's are competed then F_G = 1.0. Thus the slope of line of the plot $1/C_{pG}$ against $1/G$ in this case is equal to $H + K_G^1 c_{SG}$. By finding the value of $H + K_G^1 c_{SG}$ for each labelled preparation of E1/F8 induced RNA we can use it to evaluate F_G for all unlabelled RNA preparations competed with it.

IV. RESULTS

A. Genetical Analysis of Polar Mutants

(a) Introduction

At the present state of our knowledge of the genetic code and its mode of function we would predict that polarity must be a consequence of certain types of mutation. In the galactose operon, when all 3 enzymes are involved, the possibilities are a deletion extending from the epimerase gene through the transferase gene and into the kinase gene; an insertion of a few base pairs; a nonsense mutation in the epimerase gene which would stop translation of this and the subsequent genes; a frameshift mutation which might allow transcription and translation to proceed, but which would result in the production of inactive proteins; and combinations of these.

The possibility of polarity being due to a large deletion in the mutants under study here is unlikely since they have all been shown to produce low levels of enzyme activity on induction with fucose. (Hill and Echols, 1966; Bishop et al., unpublished work). It has also been shown that the Gal$^-$3 and Gal$^-$9 mutations of Lederberg revert spontaneously at a relatively low frequency, again excluding the deletion theory.

By examining all the mutants with respect to their spontaneous reversion frequency, response to mutagens and the types of revertants found,

we hoped to discover the cause of their polarity. The results are presented below.

(b) Spontaneous Reversion.

All the mutants used were shown to revert spontaneously. The frequency of reversion was obtained experimentally as described in the materials and methods section and are calculated as the proportion of revertants among the total viable cells grown from a single colony.

The numbers of reversions obtained are given in table 9. Gal$^-$3 was found to produce about 10 times as many revertants as the others. This value agrees with that found by Shapiro (1967) for Gal$^-$3.

(c) Response to mutagens.

The mutants were tested for their response to three types of mutagens, namely N-methyl-N^1-nitro-nitrosoguanidine(N.G.), 2-Aminopurine (A.P.) and the ICR compounds 191, 370 and 372. N.G. is a strong mutagen causing point mutations in bacteria. It has been shown to methylate guanine, adenine and cytosine in that order of preference, in tobacco mosaic virus. Methylation of guanine appears to be lethal to the viral RNA and the other reactions appear to result in mutagenesis. The mutagenic action, however, is as yet unknown.

A.P. is an adenine analogue. In its normal form it sometimes pairs with cytosine instead of thymine and like adenine, in its rare imino form it pairs with cytosine. It can act at either of two stages,

TABLE 9. Spontaneous Reversion Frequencies

Mutant	Revertants/viable cell
9-3t/119	3.5×10^{-8}
9-3t/121	2.0×10^{-8}
9-3t/156	5.0×10^{-8}
9-3t/F^{-}3	4.1×10^{-7}
9-3t/F^{-9}	5.9×10^{-8}

both of which result in transitions. When A.P. is inserted into the DNA instead of adenine it can mis-pair with cytosine at the next replication, resulting in the change AT——→ GC. If, however, AP is inserted by mis-pairing with cytosine and then subsequently pairs with thymine the opposite change occurs, i.e. CG——→ TA.

The ICR compounds have been shown to insert and delete single or small numbers of base pairs in bacteria (Ames and Whitfield, 1966; Malamy, 1966), Neurospora (Brockman and Goben, 1965) and Drosophila (Carlson and Oster, 1962; Snyder and Oster, 1964; and Southin, 1966). These are acridine-like compounds consisting of an acridine ring and an alkylating chain.

The methods of mutagenesis are described in section I of materials and methods.

Because of the strong lethal effect exhibited by nitrosoguanidine a large area of clearing is found around the crystals of mutagen on the plate. In the case of a positive response this area is bounded by a thick 'halo' of revertant colonies.

Of the five mutants tested, only the 121 mutation gave a positive result with N.G. A.P. has very little lethal effect. Thus no clear area is found on the treated plates. Again, only mutant 9-3t/121 responded.

None of the five mutants showed any increase of revertant number in the presence of the ICR compounds. The positive control in this case was, however, a frameshift mutation in a completely different genetic background. It is known that for these mutagens to act successfully, it is necessary for the bacteria to carry the rec^+ allele so that it is not unlikely that other genetic markers may play a part in the success or failure of the mutagenic ability of these compounds. Unfortunately in the time available, it was not possible to transfer the known frameshift mutation into our background. We must note, however, that other workers have failed to get known frameshift mutations (characterised by amino acid analysis) to respond to the ICR mutagens. On the basis of our negative result, therefore, we cannot definitely exclude the possibility that these polar mutants are of the frameshift type.

From these findings, however, it is clear that mutation 121 is a point mutation since it responds to both NG and A.P., whereas the remaining four do not seem to fall into this category.

(d) Analysis of Revertants.

It is well known that nonsense mutations act by terminating the translation of mRNA into protein. This is due to the absence, within the cell, of t-RNA with the anti-codon for the nonsense triplet. Such cells are termed su^-. Alleles of the su genes, however, have

been found which suppress all the known nonsense mutations. These su^+ cells contain a t-RNA which recognises the nonsense codon and inserts an amino acid at the site.

There are three known nonsense mutations, namely amber (coded for by UAG), ochre (UAA) and UGA with three corresponding classes of su^+ genes. Ochre suppressors will suppress certain amber mutations as well as ochres, but the other two types of su^+ genes are specific for their own nonsense mutations. Several su^+ alleles with different map locations are known for both ochre and amber mutations and the efficiency of action of these varies. Amber suppressors are relatively efficient giving 30-60% suppression, while ochre suppressors are much weaker, resulting in only 1-5% suppression.

Our polar mutants had previously, in this laboratory, been transferred via an F^1 into bacteria with the entire gal region deleted and carrying one or other of the ochre and amber su^+ genes. It was found that mutation 121 only was suppressed in the presence of ochre suppressors and none of the five polar mutants responded to amber suppressors. This, of course, strongly suggested that mutation 121 is an ochre type whereas 119, 156, Gal^-3 and Gal^-9 do not belong to either the ochre or amber classes.

To further test this an analysis was made of the revertants of all the polar mutants under study.

Using the technique of F-duction described in section G of the materials and methods, the F^1 gal of the revertant 9-3t/F^1 stock was transferred to 9-3h$^+$ recipient bacteria plated on selective medium. Ability to grow is conferred by a true back-mutation on the F^1. Inability to grow shows that a different gene on the revertant bacterial genome is responsible for its gal$^+$ phenotype. We interpret the latter as being a suppressor. An appropriate control was used.

The results are given in Table 10. Mutation 121 only showed evidence of suppression with approximately 15% of the revertants falling into this category. The other mutants gave only true back mutations showing that no suppressor of any type is present in these revertants.

Finally I tested all the revertants against known amber mutants of T_4 phage, one of which (Am 22) responds to ochre suppressors. The spot test method and interpretation of results are explained in material and methods section G. The results are given in table 11.

Of the revertants of mutation 121 the same ones which failed to F-duce 9-3h$^+$ in the previous experiment were found to contain an ochre suppressor. No other revertants of 121 contained suppressors. None of the revertants of 119, 156, Gal$^-$3 or Gal$^-$9 were found to suppress the T_4 nonsense mutants.

We can therefore conclude from these data that mutation 121 is an ochre nonsense type. The remaining four polar mutations under

TABLE 10. F-Duction with Revertants of Polar Mutants

Parent Stock	Number of Revertants Tested	Number Showing Gal^+F^1-Duction	Number Showing No Gal^+F-Duction	% Suppressors
9-3t$^+$/121	138	117	21	15.2
9-3t$^+$/119	58	58	0	0
9-3t$^+$/156	70	70	0	0
9-3t$^+$/F$^-$3	61	61	0	0
9-3t$^+$/F$^-$9	60	60	0	0

TABLE 11. Suppression of T-Phage Nonsense Mutants by Revertants of Polar Mutants.

Parent Stock	Number Tested	Number Containing Ochre Suppressor	% Containing Ochre Suppressor	Number Containing Amber Suppressor	% Containing Amber Suppressor
9-3t/121	138	21	15.2	0	0
9-3t/119	58	0	0	0	0
9-3t/156	70	0	0	0	0
9-3t/F$^-$3	61	0	0	0	0
9-3t/F$^-$9	60	0	0	0	0

study are not of the amber or ochre type and on the basis of F-duction experiment do not appear to be of the UGA type either. Unfortunately, it was not possible to test in more detail for UGA suppressors amongst the revertants.

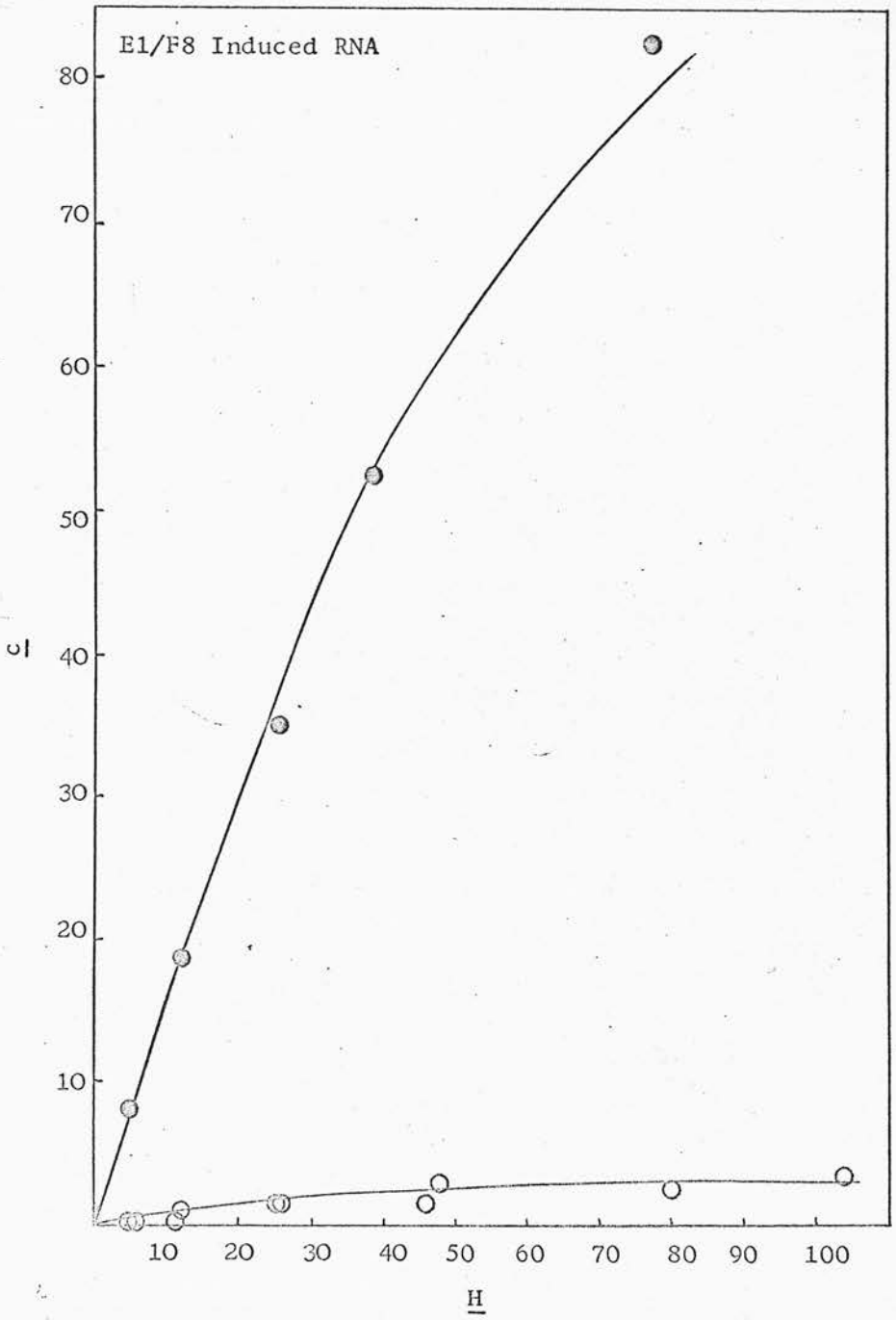
Since the gal⁻9 mutation has been shown by Jordan et al.(1968) to be the result of an insertion and the properties studied show the other mutations to act in a similar manner it is possible that at least some of these are also of this type. We can draw no conclusions about this from our results.

B. Hybridization Experiments

(a) Saturation of λ^+ DNA with various RNA preparations.

In order to examine cross-hybridization between λ^+ DNA and E.coli RNA several saturation experiments were carried out. The RNA's used were prepared from E1/F8 bacteria induced with fucose and repressed with TMG, and 9-3t bacteria induced with fucose as described in materials and methods sections O and P. Blank filters were included in the experiment for selected RNA concentrations. No non-specific radioactivity was found on these.

The results are shown in figure 9 and the experimental detail is given in the legend to the figure. The method is described in material and methods section Q.



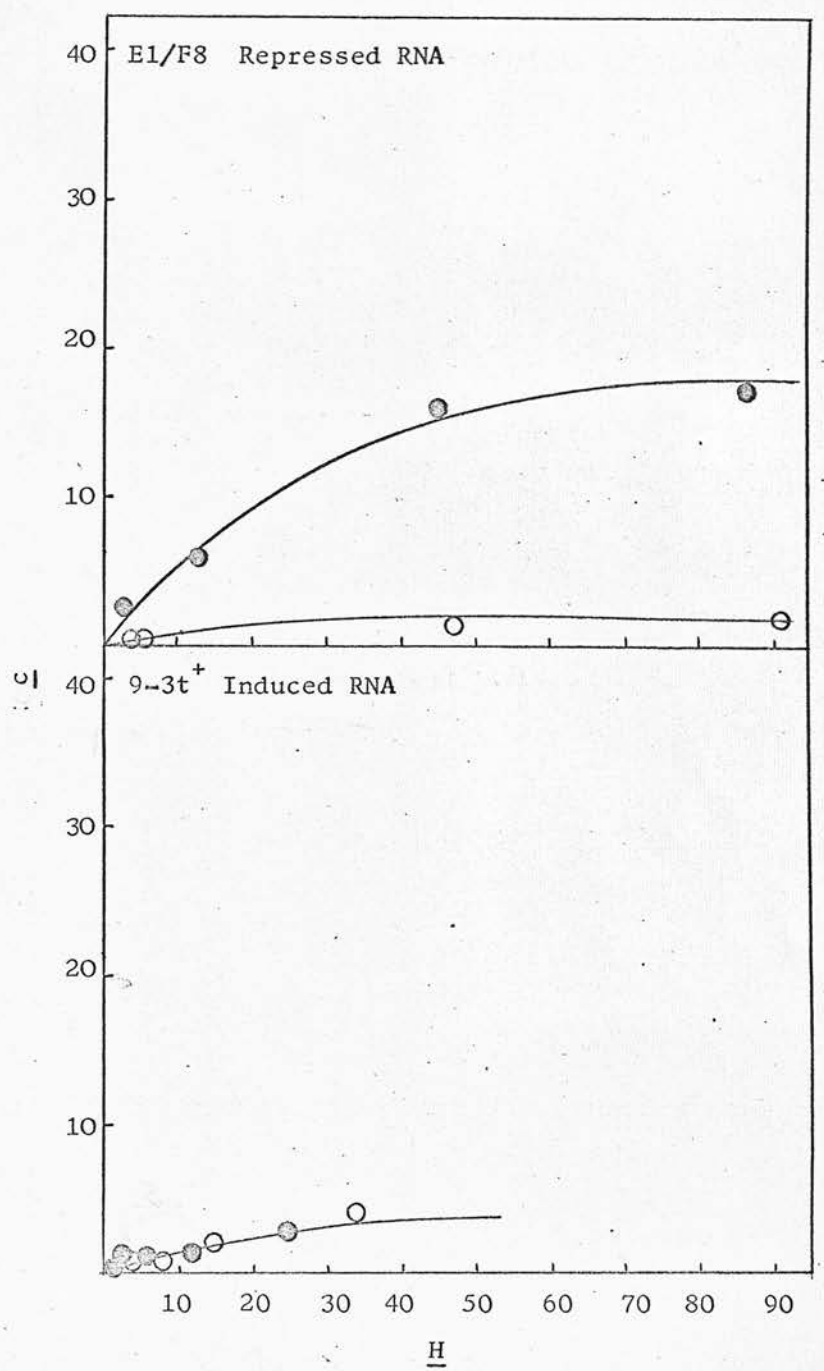


FIGURE 9. (Legend overleaf)

LEGEND TO FIGURE 9

Saturation Curves of λ^+ and λ dg 17-8 with E1/F8 Induced, E1/F8 Repressed and 9-3t Induced RNA's.

The membranes were loaded with 10 μg λ^+ or λ dg 17-8 DNA; hybridisation was carried out in 25 ml beakers each containing one membrane in 1.0 ml of standard hybridisation mix for 16 hours (see material and methods sections Q, b and c).

Counts per minute in the hybrid per μg DNA (c) is plotted against μg RNA input per μg DNA (H). All points are corrected for machine background.

o --- o hybridisation with λ^+ DNA; • --- • hybridisation with λ dg 17-8 DNA.

It will be noted that all three RNA preparations gave low levels of hybridization with λ^+ DNA and that these did not vary significantly between the RNA types. We therefore take this to be heterologous hybridization and find that the level of cross-hybridization between λ^+ DNA and E.coli RNA is very low.

(b) Saturation of λ dg 17-8 DNA with various RNA Preparations.

The preparations of RNA from E1/F8 and 9-3t used in the previous experiment were also used in saturation experiments with λ dg 17-8 DNA.

This λ dg carries the complete gal operon plus a segment of the adjacent E.coli genome (see figure 7) and should therefore hybridize with a specific fraction of the E.coli RNA. RNA from induced cultures of E.coli E1/F8 should contain both the gal and the non-gal complementary fractions; E1/F8 repressed RNA should contain at least the non-gal component; and 9-3t induced RNA should contain neither component since the deletion in these bacteria covers the entire E.coli region carried on the λ dg 17-8.

The results are shown in figure 9. It can be seen that the data correspond to the predictions made. The level of hybridization between λ dg 17-8 DNA and 9-3t RNA does not differ from that found between the same RNA and λ^+ DNA. There is a marked difference in the

saturation curves of λ^+ DNA and λ dg 17-8 DNA with the E1/F8 repressed and induced RNA's, with E1/F8 induced RNA showing a much higher level with λ dg 17-8 DNA than does E1/F8 repressed RNA.

We therefore conclude from these experiments that under the conditions used here it is possible to specifically hybridize the E.coli region of λ dg and its homologous RNA with negligible heterologous hybridization between the λ^+ DNA of the λ dg and the E.coli RNA.

(c) Saturation of λ dg 17-8 and λ dg T166 DNA's with E1/F8 Induced and Repressed RNA's.

As explained in the analysis of results section one can estimate the hybridisation between E.coli RNA and the K + T region of the gal operon by comparison of data from experiments using λ dg 17-8 and λ dg T166 DNA's. From this one calculates hybridization to the complete gal operon and can make further estimates about the gal mRNA in the preparations of RNA.

Figure 10 shows the results of typical saturation experiments using λ dg 17-8 and λ dg T166 DNA's and RNA's prepared from E1/F8 induced and repressed as described in the materials and methods sections O and P.

Taking a set of arbitary values for input RNA/μg DNA the corresponding values of c_{17-8} and c_{T166} are read from the saturation curves. The values obtained by subtraction of c_{T166} from c_{17-8} are

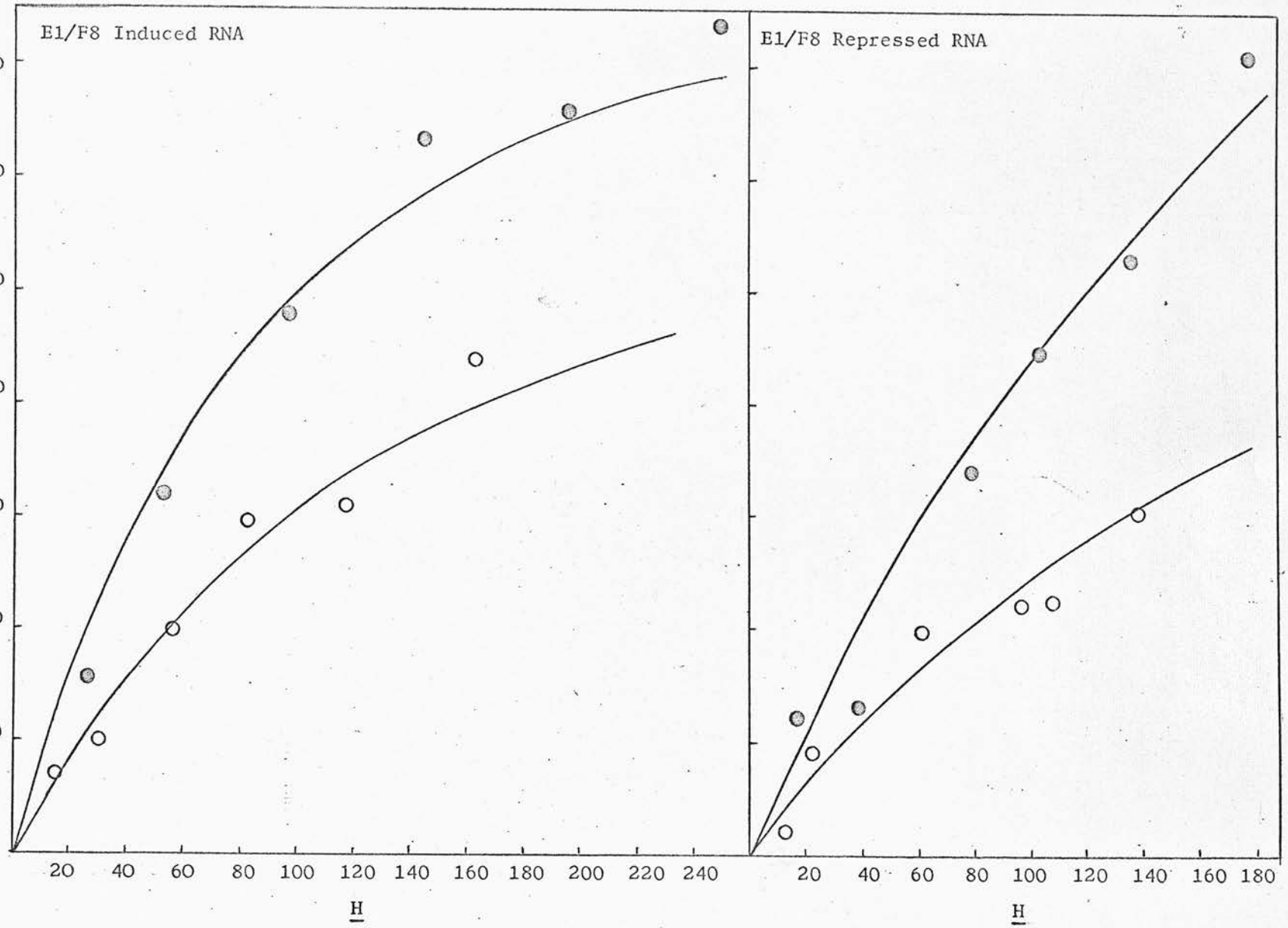


FIGURE 10. (Legend overleaf)

LEGEND TO FIGURE 10

Saturation Curves of λ dg 17-8 and λ dg T166 with E1/F8 Induced and E1/F8 Repressed RNA.

The membranes were loaded with 3 μg λ dg 17-8 or λ dg T166 DNA; hybridization was carried out in test tubes each containing 2 λ dg and 1 blank membrane in 0.7 ml of standard hybridization mix for 40 hours (see material and methods sections Q,b and c).

Counts per minute per μg DNA in the hybrid (c) is plotted agains μg RNA input per μg DNA (H). All are corrected for machine background.

● --- ● hybridization with λ dg 17-8 DNA

o --- o hybridization with λ dg T166 DNA

taken as $c_{(K+T)}$ and by multiplying these by $^3/2$ the series of estimates for c_G and subsequently c_{NG} for λ dg 17-8 DNA are obtained. The data pertaining to figure 10 are given in table 12 and the estimated saturation curves of the gal and non-gal components are shown graphically in figure 11.

By plotting $1/c_G$ against $1/H$ and $1/c_{NG}$ against $1/H$ we obtain values for K_G^1 and K_{NG}^1 respectively from the slope of the regression lines, i.e. the regression coefficients. Similarly c_{SG} and c_{SNG} are the reciprocals of the regression coefficients of H/c_G on H and H/c_{NG} on H respectively. These lines are shown in figures 12 and 13 and the values obtained are given in table 13.

The specific activity of the gal mRNA, the proportion of the total input RNA which is gal mRNA and the number of molecules of gal mRNA per cell in the two RNA preparations were calculated as explained in the analysis of results section and are shown in table 14.

By comparing the values of α for the two preparations we find that there is 6.0 times as much gal mRNA produced in induced E1/F8 cells as in repressed E1/F8 cells.

(d) Competition Experiments between Labelled E1/F8 Induced RNA and Unlabelled E1/F8 Induced and Repressed RNA's.

In these experiments, hybridization between λ dg 17-8 DNA and a constant amount of labelled E1/F8 induced RNA was examined in the

TABLE 12. Estimates of c_G and c_{NG} From Saturation Experiments Between λ dg 17-8 and λ dg T166 DNA's and E1/F8 Induced and Repressed RNA's.

E1/F8 Induced RNA						E1/F8 Repressed RNA					
H	c_{17-8}	c_{T166}	$c_{(K+T)}$	c_G	c_{NG}	H	c_{17-8}	c_{T166}	$c_{(K+T)}$	c_G	c_{NG}
50	33.0	19.0	14.0	21.0	12.0	40	21.0	11.5	9.5	14.0	7
100	50.0	31.0	19.0	28.5	21.5	80	37.5	20.5	17.0	25.5	12
150	60.0	38.0	22.0	33.0	27.0	120	50.5	28.0	22.5	34.0	16
200	66.0	44.0	22.0	33.0	33.0	160	62.0	34.0	28.0	42.0	20

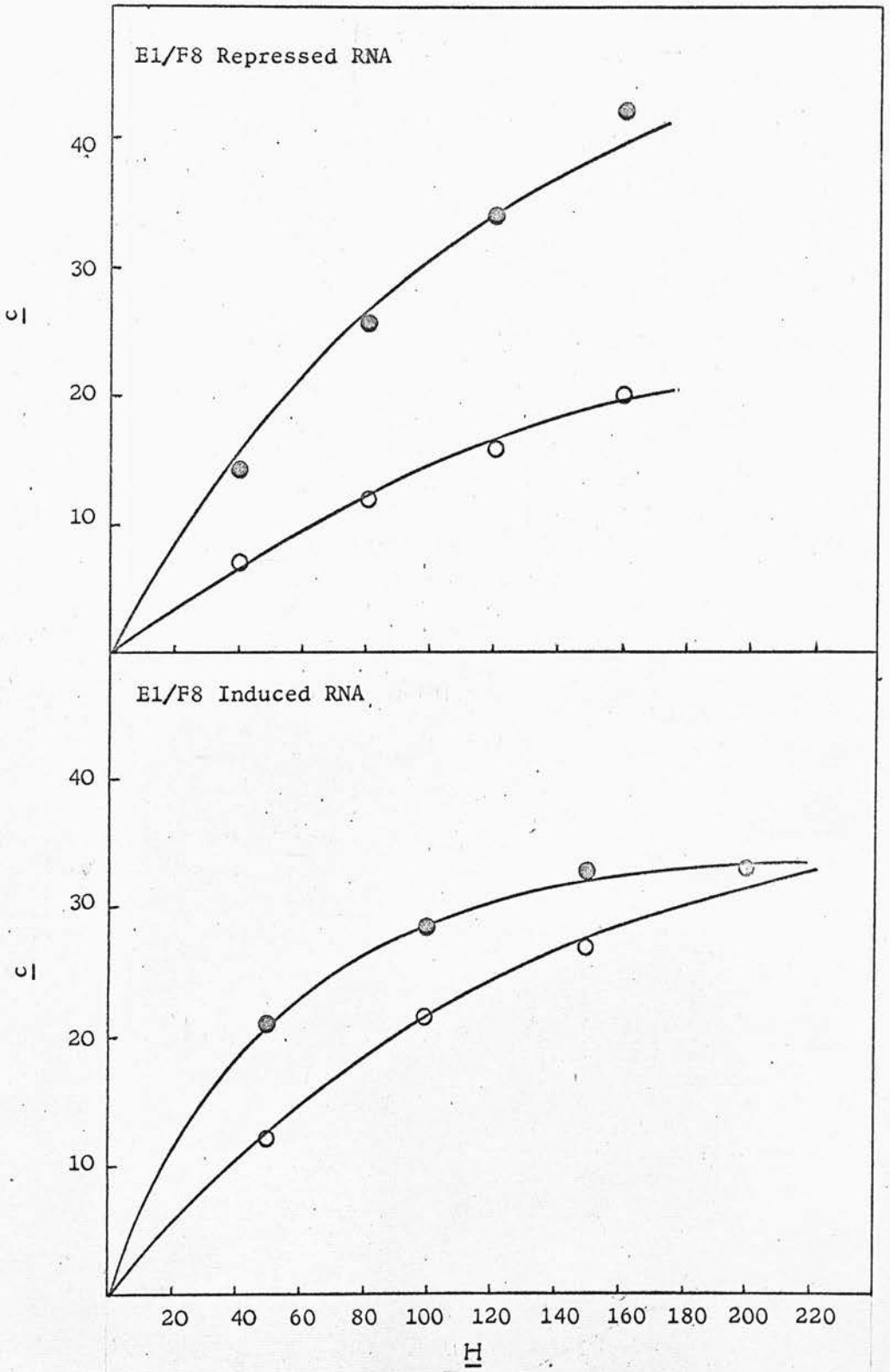


FIGURE 11. (Legend overleaf)

LEGEND TO FIGURE 11.

Estimated Saturation Curves of the Gal and Non-Gal Components E1/F8 Induced and Repressed RNA's with λ dg 17-8 DNA.

The values for c_G and c_{NG} calculated as described in analysis of results section and shown in table 12, are plotted against μg RNA input per μg DNA (H).

● --- ● Estimates of counts per minute per μg DNA in hybrid due to gal mRNA (c_G)

o --- o Estimate of counts per minute per μg DNA in hybrid due to non-gal RNA (c_{NG}).

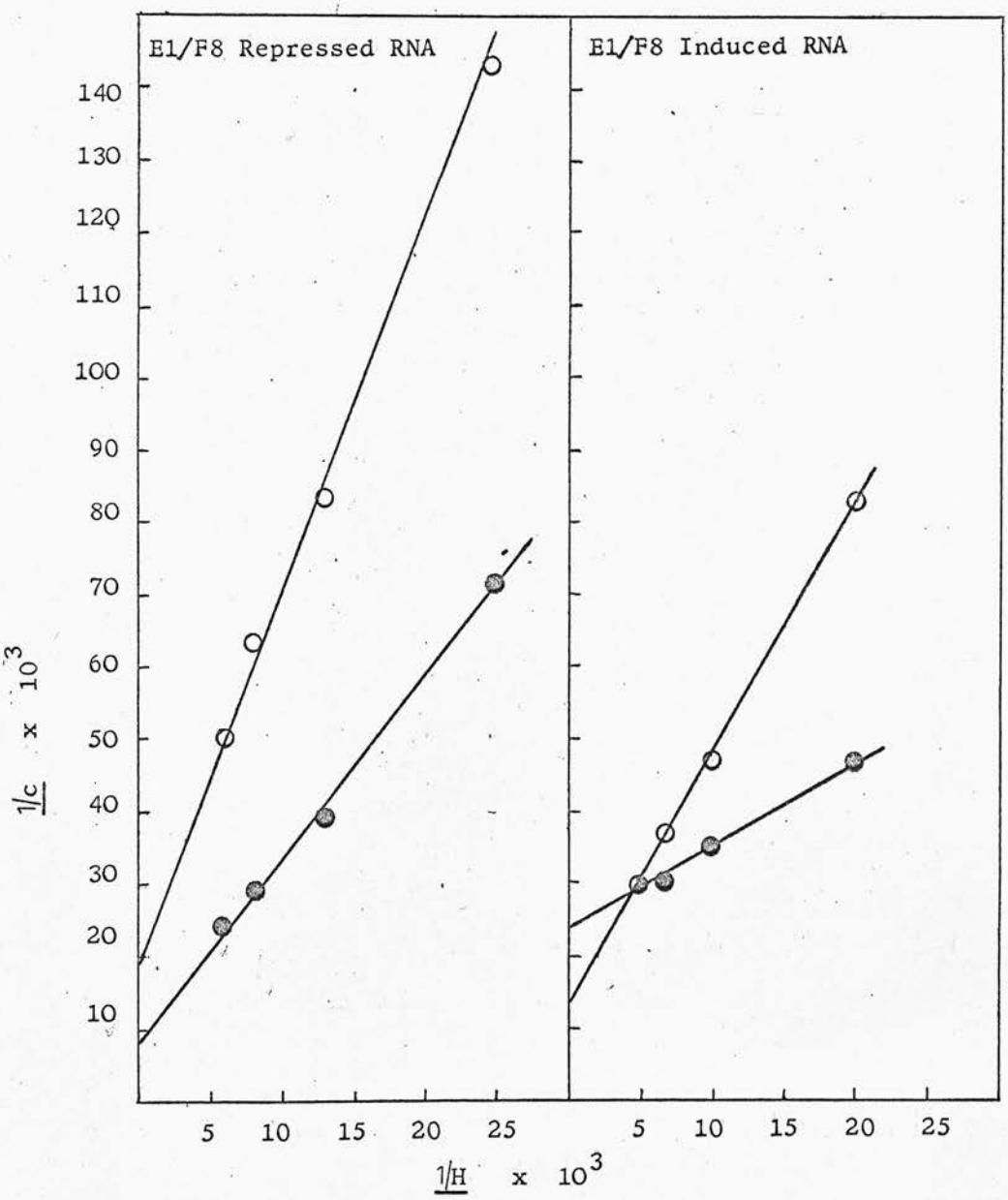


FIGURE 12. Reciprocal Plots of 1/c against 1/H for Gal and Non-Gal Components of E1/F8 induced and repressed RNA's hybridised to λ dg 17-8 DNA

The values of $1/c_G$ and $1/c_{NG}$ are the reciprocals of the estimates of c_G and c_{NG} shown in table 12.

o --- o $1/c_{NG}$ versus $1/H$

● --- ● $1/c_G$ versus $1/H$

The reciprocal of the regression coefficients give the values of K_G^1 and K_{NG}^1 for the two preparations. These are shown in table 13.

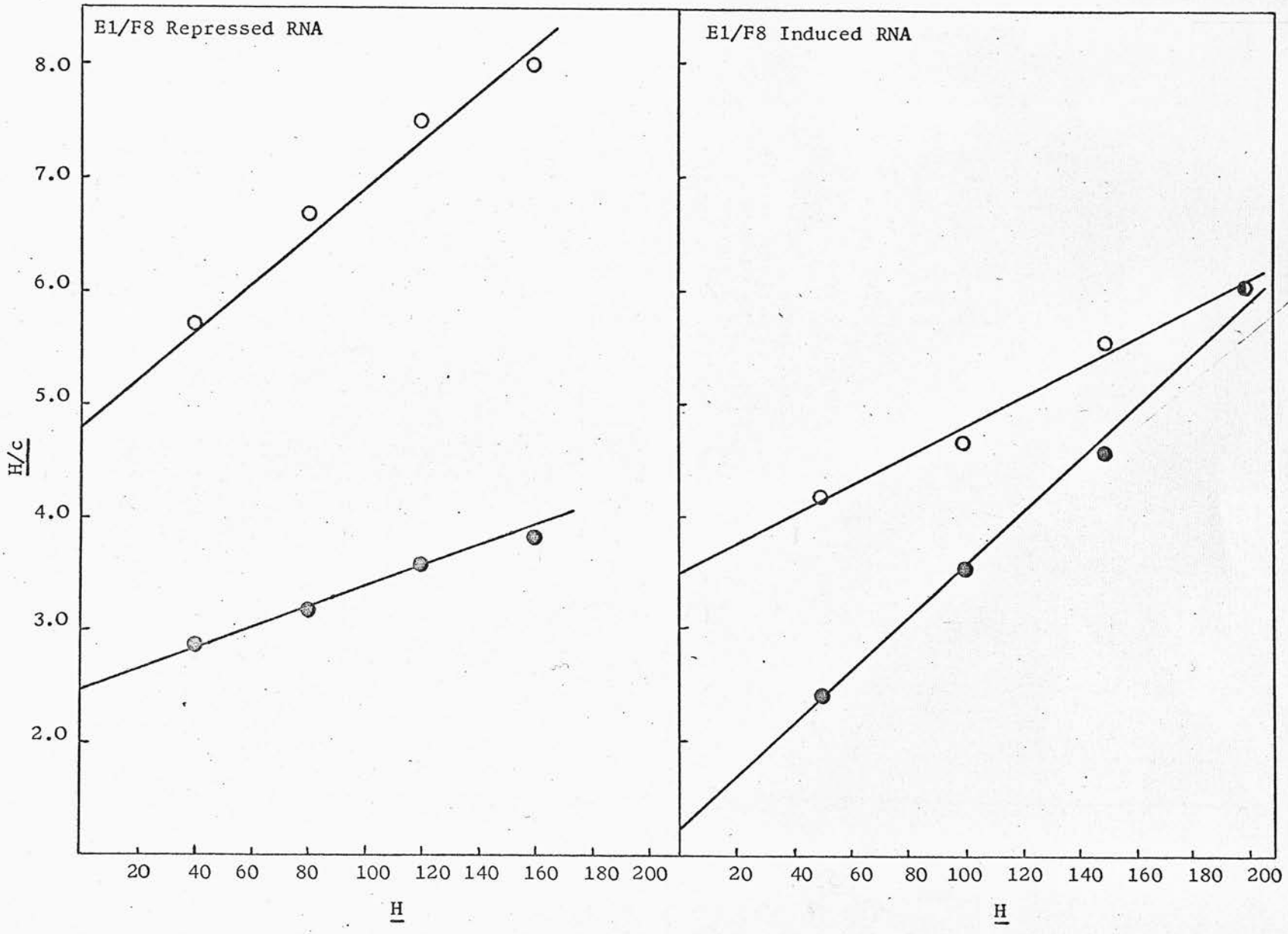


FIGURE 13. (Legend overleaf)

LEGEND TO FIGURE 13

Estimated values of H/c for Gal and Non-Gal Components of E1/F8 Induced and Repressed RNA's Hybridized to λ dg 17-8 DNA plotted Against Input RNA (H).

The values of H/c for the gal and non-gal components are calculated as described in the analysis of results section using the estimated values for c_G and c_{NG} obtained from the saturation experiments with λ dg 17-8 and λ dg T166 DNA's and shown in table 12. These are plotted agains μg input RNA per μg DNA (H).

o --- o H/c_{NG} versus H

● --- ● H/c_G versus H

The reciprocal of the regression coefficients give the values for c_{SG} and c_{SNG} for the two preparations. These are shown in table 13.

TABLE 13. Values of c_{SG}, c_{SNG}, K_G^1 and K_{NG}^1 From Saturation of λ dg 17-8 and λ dg T166 DNA's with E1/F8 Induced and Repressed RNA's.

RNA	c_{SG}	c_{SNG}	K_G^1	K_{NG}^1
E1/F8 Induced	41.39 ± 2.43	75.99 ± 6.64	1.19 ± 0.09	3.51 ± 0.007
E1/F8 Repressed	123.07 ± 6.84	51.95 ± 5.20	2.47 ± 0.12	4.82 ± 0.17

TABLE 14. Values of s.a., α and, m of E1/F8 Induced and Repressed RNA's From Saturation Experiments with λ dg 17-8 and λ dg T166 DNA's

RNA	s.a.	α	m
E1/F8 Induced	2956	2.8×10^{-4}	29.12
E1/F8 Repressed	8791	4.6×10^{-5}	4.78

s.a. = Specific activity of gal m RNA (counts per minute per μg)

α = Proportion of total input RNA which is gal mRNA

m = Number of gal mRNA molecules per cell

presence of increasing amounts of cold RNA, prepared from E1/F8 grown under induced or repressed conditions. The input of labelled E1/F8 RNA per μg DNA was calculated precisely for each hybridisation vessel.

The proportion of competition (Cp) at each input of competitor was calculated from the formula $C_p = \frac{c_o - c}{c_o}$ as explained in the analysis of results section. These values were plotted against input of unlabelled RNA per μg DNA and the resulting competition curves for E1/F8 induced and repressed competitors are shown in figure 14A. Experimental detail is given in the legend to the figures and in the material and methods section.

As expected, the induced E1/F8 RNA is seen to act as competitor more efficiently than the repressed E1/F8 RNA.

Estimates for the cpm in the hybrid due to the non-gal RNA component were calculated for each input value of unlabelled competitor using the formula

$$c_{NG} = \frac{Hc_{SNG}}{K^1 c_{SNG} + H + G}$$

as explained in the analysis of results section. The c_s value of the labelled RNA had previously been found from saturation experiments. By subtracting the estimated values of c_{NG} from the observed total cpm/μg DNA in the hybrid at the same input of competitor the corresponding series of estimates for c_G was obtained. The proportion of competition

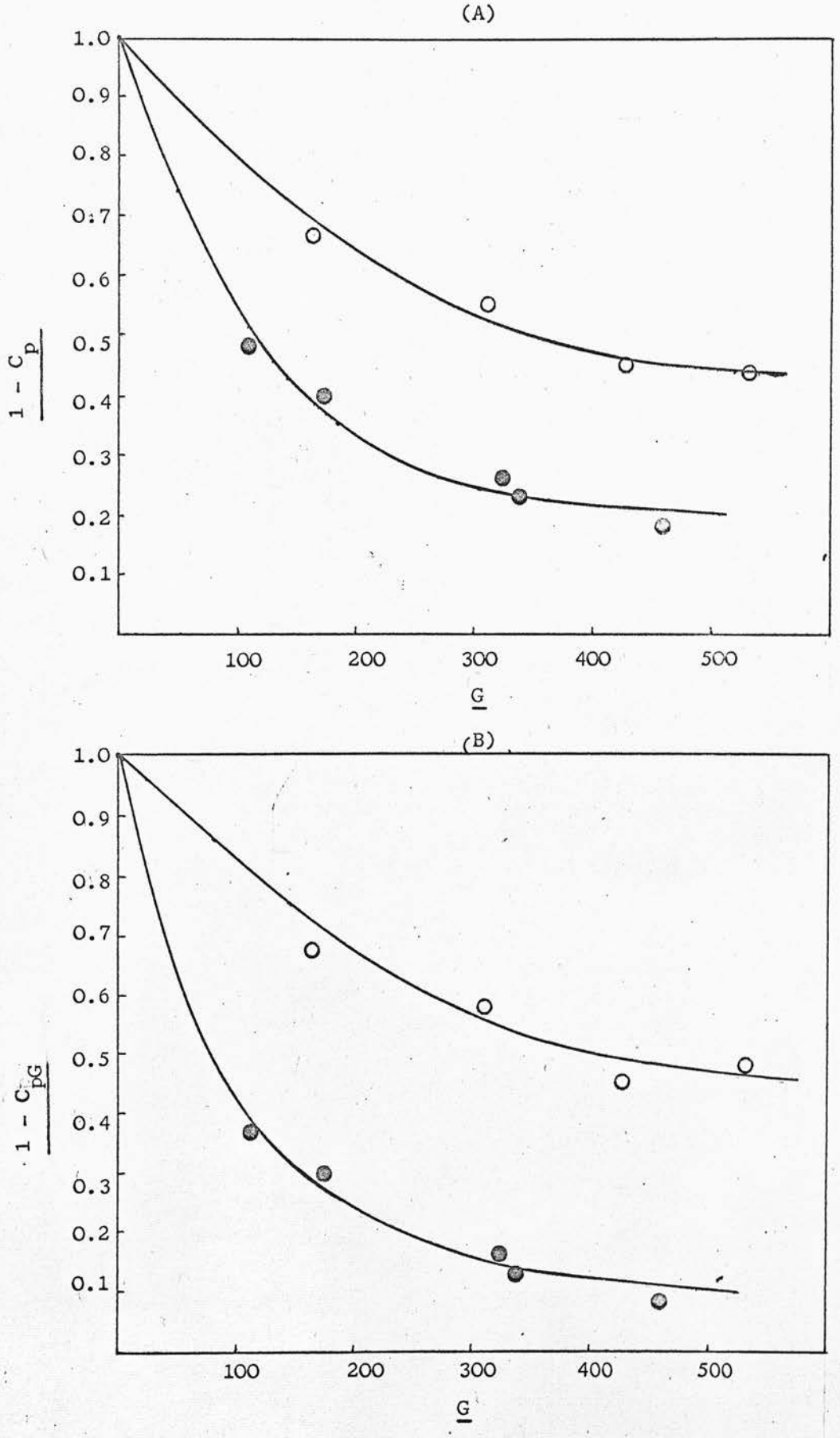


FIGURE 14. (Legend overleaf)

LEGEND TO FIGURE 14

Competition Curves Between C^{14} E1/F8 Induced RNA and Unlabelled E1/F8 Induced and Repressed RNA's. Hybridised to λ dg 17-8 DNA.

Hybridization was carried out in the standard hybridization mix containing 334 μg of C^{14} E1/F8 induced RNA and increasing amounts of unlabelled E1/F8 induced or repressed RNA's, in a total volume of 0.7 ml in tubes. Each tube contained 2 λ dg 17-8 membranes loaded with approximately 4.5 μg DNA and 1 Blank membrane, and the incubation time was 40 hours at 65°C. A control tube containing no cold RNA was included in the experiment and the proportion competition was calculated taking this as zero competition. ● --- ● E1/F8 Induced o --- o E1/F8 Repressed.

(A) $1-C_p$ = Proportion of total counts remaining in hybrid plotted against input unlabelled E1/F8 induced or repressed RNA in μg/μg DNA.

(B) $1-C_{pG}$ = Proportion of gal component counts remaining in hybrid plotted against input unlabelled E1/F8 induced or repressed RNA in μg/μg DNA. C_{pG} is calculated as described in the text.

by the gal mRNA component was calculated for each input of unlabelled RNA using the formula $C_{pG} = \frac{C_{OG} - C_G}{C_{OG}}$ (see analysis of results section). The competition curves for the gal mRNA with E1/F8 induced and repressed competitors are shown in figure 14B.

The reciprocal of proportion competition is plotted against the reciprocal of µg cold competitor per µg DNA added (figure 15). The line through the points is forced through $1/C_{pG} = 1.0$ at $1/G = 0$ since we assume that p = 1.0 and the intercept of the Y axis of this plot = 1/p, as explained in analysis of results section. Since the relative strength of gal mRNA in hot and cold E1/F8 induced RNA must equal 1.0 (i.e. F = 1.0), the slope of the reciprocal plot of competition experiments between these must equal $K_G^1 c_{SG} + H$. By substituting this value for $K_G^1 c_{SG} + H$ and p = 1.0 in the equation

$$\text{Slope of reciprocal plot} = \frac{F_G(K_G^1 c_{SG} + H)}{p}$$

the value of F_G for E1/F8 repressed RNA was obtained.

For E1/F8 repressed RNA, F_G is found to equal 6.0. Thus the concentration of gal mRNA in a repressed preparation of E1/F8 is 6 times less than in an induced preparation. This agrees well with the findings using data from saturation experiments.

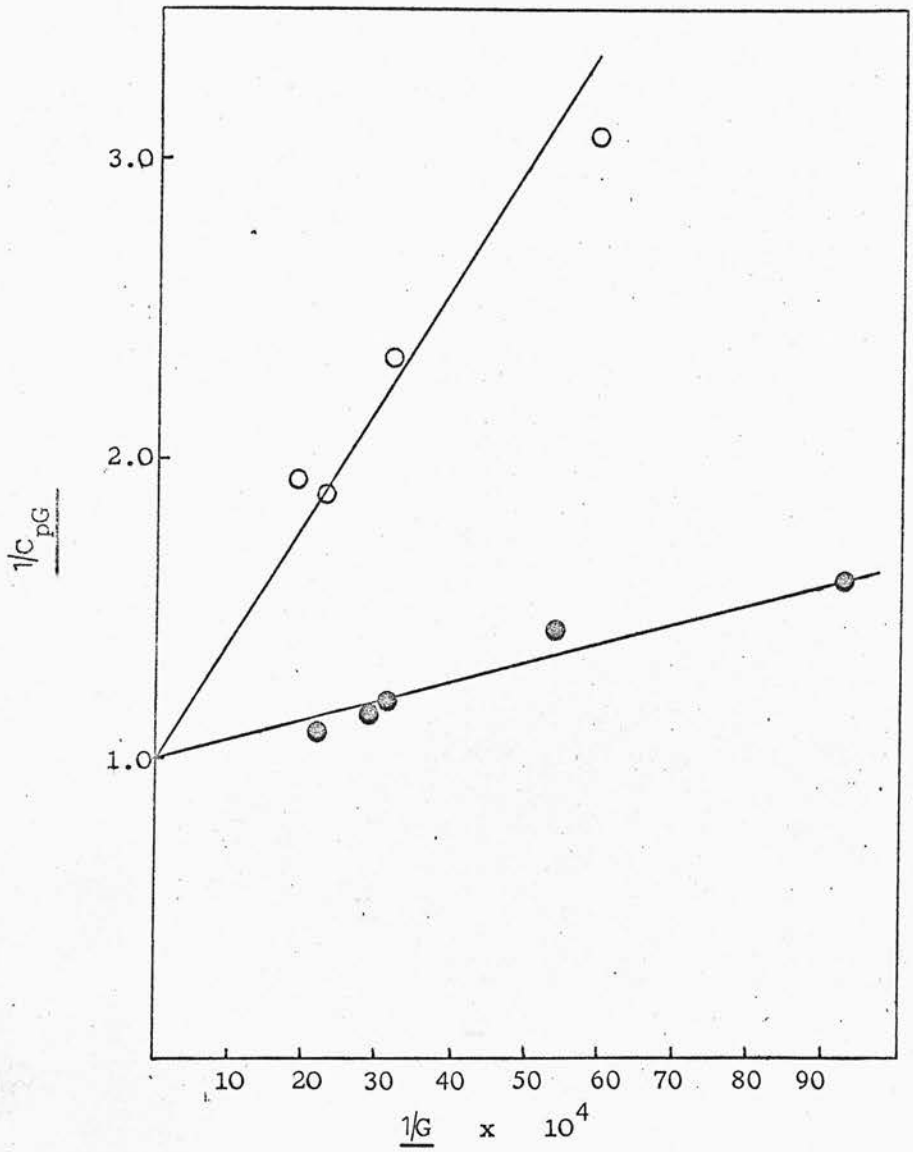


FIGURE 15. Reciprocal plots of $1/C_{pG}$ against $1/G$ for Competition Between C^{14} Induced RNA and Unlabelled E1/F8 Induced or Repressed RNA's

The reciprocal of the proportion competition of the gal component ($1/C_{pG}$) of E1/F8 induced and repressed RNA's are plotted against the reciprocal of the input of unlabelled competitor in μg/μg DNA $1/C_{pG}$ is calculated as described in the text.

The lines through the points are forced through $1/C_{pG} = 1.0$ at $1/G = 0$ in order to determine the value of F_G for E1/F8 repressed from comparison of the slopes.

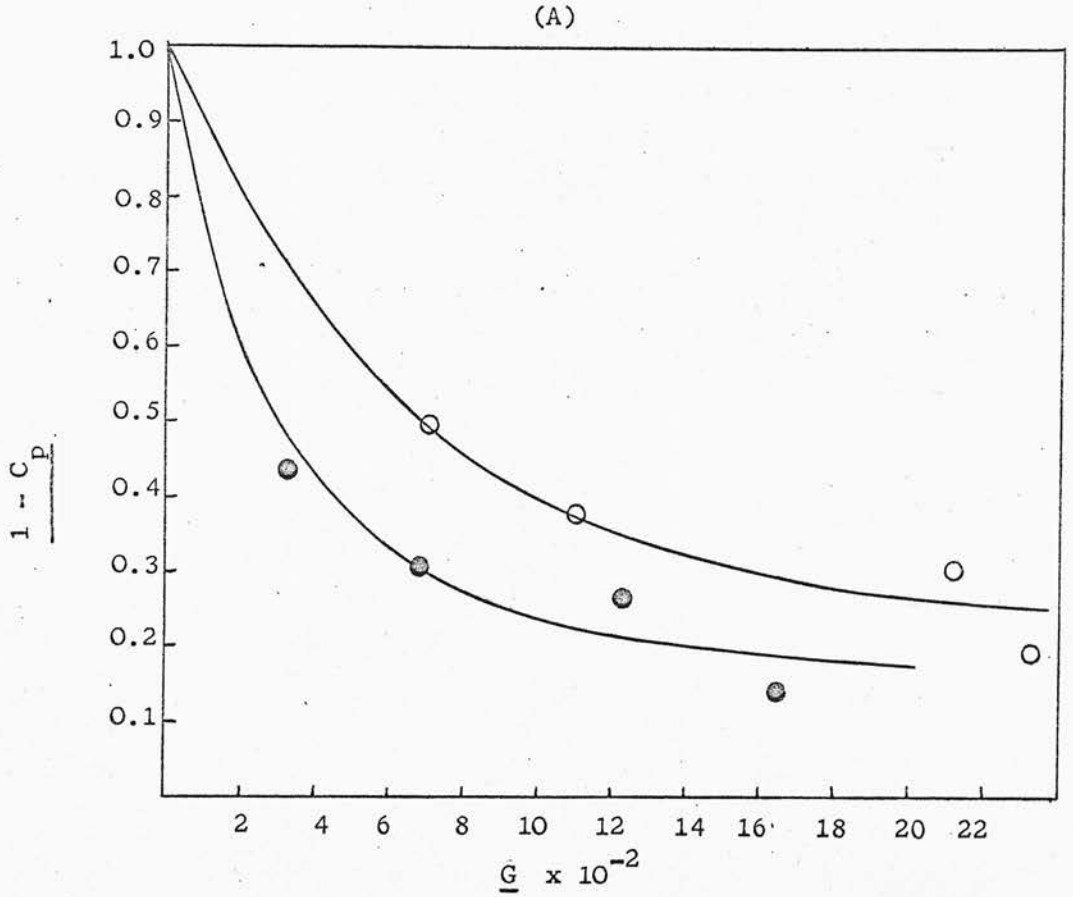
● --- ● E1/F8 Induced o --- o E1/F8 Repressed

(e) Competition Experiments between Labelled E1/F8 Induced RNA and Unlabelled E1 Induced RNA.

In order to obtain a rough estimate of the extent of the error in our data and method of analysis I took RNA prepared from E.coli E1 induced with fucose and used it as competitor for labelled E1/F8 induced RNA. E1/F8 contains approximately 2.5 gal operons per cell whereas E1 carries only one gal operon on the bacterial genome. The gal enzyme produced by E1 induced bacteria is 2.5 times less than that produced by E1/F8 induced bacteria. Since no type of regulation mechanism is operating in this case we can therefore predict that gal mRNA will also be 2.5 times less in E1 total RNA than in E1/F8 RNA prepared from cultures grown under identical conditions.

A homologous competition experiment between labelled and unlabelled E1/F8 induced RNA's was again carried out to obtain our value for $K^1 c_{SG} + H.c_S$ for the labelled E1/F8 preparation was found by saturation experiments.

The competition curves are shown in figure 16A, and the experimental detail given in the legend to the figure. The competition due to the gal mRNA component, calculated as described in the previous section and in the analysis of results section is given in figure 16B, and the reciprocal plot is shown in figure 17.



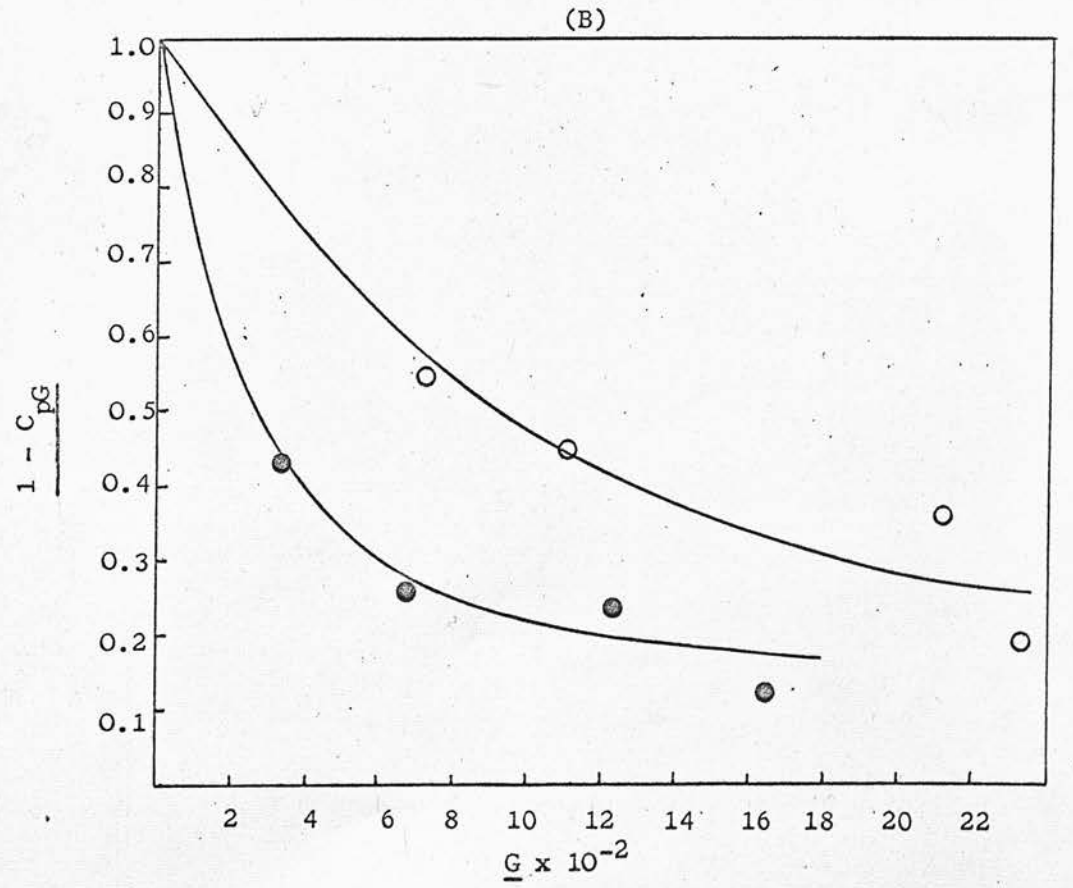


FIGURE 16. (Legend overleaf)

LEGEND TO FIGURE 16

Competition Curves Between C^{14} E1/F8 Induced RNA and Unlabelled E1/F8 and E1 Induced RNA's Hybridized to λ dg 17-8 DNA.

Hybridization was carried out as described in the legend to figure 15. • --- • E1/F8, o --- o E1.

(A) $\underline{1-C_p}$ = Proportion of total counts remaining in hybrid plotted against input unlabelled RNA in μg/μg DNA. (G).

(B) $\underline{1-C_{pG}}$ = Proportion of gal component counts remaining in hybrid plotted against input unlabelled RNA in μg/μg DNA (G). $\underline{C_{pG}}$ is calculated as described in the text.

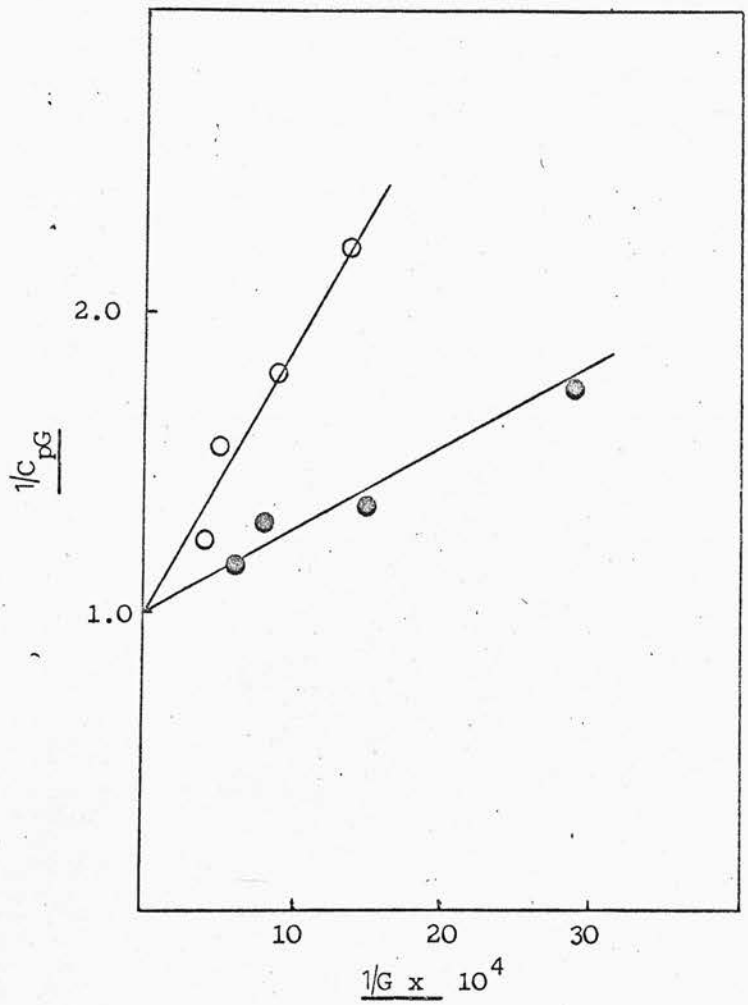


FIGURE 17. Reciprocal Plots of $1/C_{pG}$ against $1/G$ for Competition between C^{14} E1/F8 Induced and Unlabelled E1/F8 and E1 Induced RNA's.

The reciprocal of the proportion competition of the gal component ($1/C_{pG}$) of E1/F8 and E1 induced RNA's are plotted against the reciprocal of the input of unlabelled competitor in μg/μg DNA. $1/C_{pG}$ is calculated as described in the text.

The lines through the points are forced through $1/C_{pG} = 1.0$ at $1/G = 0$ in order to evaluate F_G for E1 induced RNA.

● --- ● E1/F8 induced o --- o E1 induced

The slopes of the lines forced through $1/C_{pG} = 1.0$ at $1/G = 0$ are 275.0 for E1/F8 induced and 875.0 for E1 induced competitors which gives F_G value for E1 induced of 3.18.

$F_G = 3.18$ differs from the expected 2.5 by less than 30% . Thus it is estimated that the margin of error in the results for the F_G value are within 30% of the true value.

(f) Competition Experiments between Labelled E1/F8 Induced RNA and Unlabelled RNA Prepared from Induced Cultures of the Polar Mutants NA 119/119, NA 121/121, NA 156/156, F Gal$^-$3 and F Gal$^-$9.

These experiments were carried out in groups in each of which a single preparation of labelled E1/F8 induced RNA was competed out in different experiments by different mutant RNA preparations. The experimental technique was identical to the competition experiments previously reported. Homologous hot and cold induced E1/F8 RNA's were competed in each group to obtain the value of K^1c_{SG}+ H and the c_S value of every labelled preparation was found from saturation experiments.

Table 15 gives the values of F_G found for all the polar mutants. Duplicate experiments gave values of F_G which were all within 30% of each other.

It appears from these results that all the polar mutants ~~transcribe~~ synthesise gal mRNA to a considerable extent. F Gal$^-$3 has the least

TABLE 15. The Relative Concentration of Galactose mRNA in Labelled E1/F8 Induced RNA and Polar Mutants NA119/119, NA121/121, NA156/156, F Gal^{-}3 and F Gal^{-}9 Induced RNA's.

Bacterial Source of RNA As Competitor	Growth Conditions	F_G	Gal mRNA as Percentage of E1/F8 Induced
NA119/119	Induced	2.81	35.6
NA121/121	Induced	2.22	45.0
NA156/156	Induced	3.33	30.0
F Gal^{-}3	Induced	4.00	25.0
F Gal^{-}9	Induced	1.59	62.9

The labelled RNA in all cases was E1/F8 induced with fucose.

F_G = The relative concentration of galactose mRNA in labelled and unlabelled RNA's.

gal mRNA with a 4 times lower concentration than E1/F8 grown under the same conditions, while F Gal^{-}9 has only 1.6 times less. The polar mutants isolated in this laboratory all fell between these two limits.

V. DISCUSSION

(a) Induction and Repression of the Gal Operon

The technique of hybridization of RNA from cells grown under different physiological conditions to DNA of a more specific nature than total cellular DNA has been used to examine mRNA of various operons by many workers. To date, it has been found that an increase in the amount of hybrid formed and therefore, presumably, of the operon specific RNA in the cells is found when operons are induced or derepressed compared to that found when they are repressed or uninduced. The gal operon is no exception to this.

Attardi et al.(1962, 1963a and b) and Hill and Echols (1966) showed a substantial increase in gal mRNA (as measured by hybridization of the RNA to λ dg DNA) from cells induced with the gratuitous inducer, fucose, over cells grown on glycerol (uninduced) or glucose (repressed) as carbon source. Using T.M.G. as repressor we have shown that RNA from cells grown in the presence of T.M.G. contains much less gal specific RNA than RNA from induced cells.

The experimental design commonly used for these experiments is to compare the hybrid formed between only one level of the different RNA's estimated in radioactivity and excess DNA. In the course of this work, however, I have carried out saturation experiments which enable one to calculate the saturation value for different RNA's.

The source of gal DNA used here as well as that used by Attardi et al. and Hill and Echols was a λ dg which is known to carry the E.coli region between the λ^{att} site and the gal operon. That this region is transcribed is suggested by the work of Guha et al. (1968). They found that the increase in hybrid formed between gal DNA and RNA from induced cells was due to hybridization to the less dense (W) strand of the DNA duplex. However, a low level of hybridization was consistantly found between RNA of both induced and uninduced cells with the denser (C) strand of the gal DNA, and the amount did not increase upon induction. They interpreted this to mean that gal mRNA is ~~transcribed~~ synthesised from the W strand but that there is RNA in the cells which is homologous to the λ^{att}-gal region of DNA on the λ dg. I was able to eliminate hybridization to this non-gal region from my results by utilising the incomplete gal phage λ dg T166. Unfortunately, however, I was unable to compensate for any hybridization to non-gal DNA carried distal to the gal operon. My estimates of hybridization to the gal operon DNA, however, I believe to be more accurate than RNA hybridized to the complete λ dg which has always been taken previously as hybridization to the gal operon.

From these estimates of hybrid formed with gal DNA one is able to calculate the specific activity of the gal mRNA. As can be seen from table 13, the specific activity of gal mRNA in different preparations is found to vary greatly, thus making suspect any

quantitative conclusions from data given in units of radioactivity or obtained from experiments where equal amounts of radioactivity rather than equal amounts by weight of RNA are added for comparison. To overcome this problem in data presented here I calculated from the saturation values the proportion of gal mRNA in the total RNA preparations used and was thus able to compare directly the amount of gal mRNA present under the different conditions.

Using saturation data and our method of calculation a 6-fold difference between the levels of gal mRNA from cultures grown in the presence of fucose and glycerol plus T.M.G. was found.

By using competition experiments one is able to eliminate directly any discrepancies in the results due to differences in the specific activities of gal mRNA in different preparations of RNA, since the effects of different unlabelled RNA's on hybridization of a single labelled RNA are assessed. Another advantage of both types of experiment is that in competition experiments one is comparing the effect of the total specific RNA rather than that of the fraction into which radioactive label has been incorporated, during the short labelling period. If the rate of transcription is affected by repression and induction then confusion could arise in the interpretation of saturation data which is assumed to measure mRNA level.

In fact we find a remarkable agreement between results from saturation and competition experiments. A difference of 6-fold in

specific gal mRNA was again found between induced and T.M.G. repressed preparations from the competition data.

In this laboratory it has been found that there is a 30-fold difference in the kinase and transferase enzyme activities between fucose induced cells and those repressed by either glucose or T.M.G. A similar result for glucose repressed cells has been quoted by Attardi et al. accompanied by a 9-16 fold difference in specific mRNA. Although we must bear in mind that the results of Attardi et al. for RNA are obtained by direct comparison of the radioactivity in the specific hybrids it is obvious that neither they nor ~~us~~ we find a quantitative correlation between mRNA levels and enzyme activities under the respective physiological conditions. Likewise Hill and Echols and Attardi et al. do not find a quantitatively correlated response in induced and uninduced cells.

Hybridization to E.coli genes distal to the gal operon on the λ dg DNA would presumably have a disproportionate effect on our saturation data with induced and repressed RNA's owing to their being independent of the control of the gal operon. This has not been taken into account in the calculations of the saturation data presented here. The competition data, however, is not subject to this criticism thus the six-fold difference between the levels of RNA's from induced and repressed cells would appear to be due to gal specific mRNA.

A quantitative correlation between levels of mRNA and enzyme activity are a prerequisite for placing the regulation of an operon at the level of transcription. Such a quantitative correlation has not been found by me in the gal operon.

The data presented here suggests either that regulation of the gal operon occurs primarily at the level of translation and that the increase in specific mRNA on induction is a secondary consequence of this or that regulation occurs both at the transcriptional and translational levels.

It is difficult to envisage how translational control alone would operate, particularly in the face of evidence from other operons. The specific binding of the λ and lac repressor substances to their respective operator sites on the DNA (Gilbert and Muller-Hill, 1967; Riggs et al., 1968) conclusively shows that, as predicted, the operator is the site of repressor action, at least in these cases. The gal operon has a well defined operator site as shown genetically using the same criteria as those for λ and lac. This would, therefore, suggest that the gal repressor should also exert its controlling function from the operator site of the DNA.

Imamoto (1970) has demonstrated that transcription of the first gene of the trp operon ceases within 20 seconds under conditions where transcription of the complete operon takes 8 minutes. Such a rapid response favours the idea that transcriptional arrest is the primary reaction of the repressor since one would expect a secondary

response to take longer for expression.

A simple mechanism whereby control of the operon could primarily operate at the level of translation whilst appearing to act by attaching to the operator site would be one in which the repressor masks the protein synthesis initiation site. The specific masking effect could be achieved by attachment to the operator site of the DNA and subsequent detachment of repressor with the newly transcribed mRNA. We could then further postulate that the repressor detaches from the mRNA removing at least one base of the initiation codon or that the premature breakdown of the untranslated mRNA is unimpaired by the presence of the repressor molecule. The operator-attached repressor molecule would not be influenced by inducer and thus the lag found on induction would be due to synthesis of repressor-free mRNA and its translation into active protein.

The only work on decay of mRNA under different physiological conditions reported in the literature, however, does not support the idea of rapid degradation of messenger during repression. Lávallé and De Hauwer (1970) demonstrated, by kinetic hybridization studies, that trp mRNA from repressed cells decays at the same rate as that from cells grown in the absence of tryptophan.

A combination of transcriptional and translational control has been postulated for the trp operon (Lavallé and De Hauwer, 1970).

These authors found no quantitative correlation between enzyme and trp mRNA levels in cells grown under repressed and derepressed conditions. The also found differences between the kinetics of enzyme and mRNA production during repression and derepression. This and their results with the inhibitor of transcriptional initiation, rifampycin, led them to conclude that trp mRNA translation is regulated under repressed conditions. They postulated that this control is exerted by a molecule whose concentration is built up during derepression and which severely inhibits translation in the presence of tryptophan.

Whether or not a similar mechanism operates in the gal operon cannot be guessed at from the results reported here. From this data, however, we can conclude that regulation of the gal operon is more complex than the transcriptional control postulated by Jacob and Monod (1961a and b).

(b) Polar Mutants

From the genetic data on the polar mutants, NA 119, NA 121, NA 156, Gal$^-$3 and Gal$^-$9 it is found that only NA 121 is of the point mutation type. By F-duction and suppression tests of its revertants I have shown that in NA 121 the base change has resulted in the production of an ochre nonsense triplet.

Of the others examined no positive conclusions can be drawn from the genetic analysis alone. None are point mutations since they do not respond to the mutagens N.G. or A.P., and they do not appear to be

of the frameshift type as judged by their lack of reversion with ICR compounds. I do not feel, however, that I can completely exclude the frameshift hypothesis since the negative result could be due to the genetic background in which the mutants were carried and also because of the possibility of "hot spots" for ICR mutagenesis. Newton (1970) has demonstrated that mutagenesis of the lac operon with ICR 191D results in a series of "hot spots" clustered mainly at the operator proximal end of the gene, rather than dispersed throughout. The selective nature of the ICR mutagens may thus exclude reversion of the mutations studied here.

It is known that the Gal$^-$9 mutation is caused by the insertion of a number of bases (Jordan, Saedler and Starlinger, 1968). In my genetical analysis Gal$^-$9 behaved similarly to the other mutations. Thus it seems possible that at least some of these might also belong to this category. Shapiro (1969) reported that a high proportion of spontaneous gal polar mutations had the properties of insertions and indeed, demonstrated by density centrifugation that four extremely polar transferase mutants had a density greater than the gal$^+$ wild type. His criteria were extreme polarity, low reversion frequency, lack of reversion by base analogue or framshift mutagens and failure to respond to external suppressors. The mutations under study here, NA 119, NA 156 and Gal$^-$3 all exhibit these properties adding weight to the possibility that they are insertions.

Assuming that NA 119, NA 156 and Gal$^-$3 as well as Gal$^-$9 do carry insertions, however, the question of how this causes polarity still remains. The alternatives open to discussion at the present moment are that the insertion contains a nonsense codon, causes a shift in the reading frame of the mRNA or contains a signal for the termination of transcription.

If a transcriptional stop were the explanation one would assume that no mRNA would be ~~transcribed~~ synthesised beyond this point, and thus no proteins would be made. As can be seen from the hybridization data presented here (table 15) substantial amounts of gal specific mRNA are detected in all the mutant cells. In addition low levels of kinase and transferase are found in all cases. To overcome these facts we must further postulate a second polymerase initiation site (promotor) either at the beginning of the transferase gene, between the epimerase and transferase genes or within the insertion itself. Evidence of internal promotor sites has been found in the histidine operon of Salmonella typhimurium (Aitkins and Loper, 1969) where two such sites are implicated. These may be at the beginning of the relevant cistrons or in a non-translated region between them, in what has been termed the intercistronic divide by Rechler and Martin (1970). Such an intercistronic divide has been demonstrated by Rechler and Martin (1970) in the histidine operon. They have shown that between the translation termination codon of cistron D and the initiation codon of cistron C

there is at least one base. The same could be true of the gal operon of E.coli, thus a second internal promotor could be present in the transferase gene or between it and the epimerase cistron.

If a transcriptional stop sequence, followed by a second promotor site were the case then one would expect a quantitative correlation between the difference in levels of mRNA and enzyme activities between mutants and wild type. In this laboratory the enzyme activities of the mutants expressed as a percentage of wild type are as follows:

NA 119	20%
NA 156	5%
Gal^-3	< 1%
Gal^-9	< 1%

Hill and Echols (1966) report a similar result for Gal^-3.

As seen from table 14 the levels of mRNA in the mutants expressed as a percentage of wild type are higher than the levels of enzyme activity. Hill and Echols (1966) also found the gal mRNA level for Gal^-3 to be a higher percentage (22%) of wild type than the level of enzyme activity. Our results and those of Hill and Echols for mRNA determination cannot be directly compared as explained in the previous section of the discussion, but it is obvious that they are qualitatively in agreement.

In the case of mutant NA 119, however, the 30% error calculated for my values of F_G would allow reasonable quantitative agreement between levels of mRNA and enzyme activity. This mutation might, therefore, be the result of a transcriptional stop followed by a promotor of lower efficiency than that found at the beginning of the operon. The position of the second promotor could not be guessed at.

No quantitative correlation can be claimed for NA 156, Gal^-3 or Gal^-9 where the mRNA is found to be vastly in excess of active enzymes translated from it. The data for these mutations suggest a mechanism acting primarily at the level of translation and thereby affecting the levels of mRNA detected in the cells.

Let us consider the situation if the inserted nucleotide sequence contained a nonsense codon. Translation of the mRNA would terminate at the site of the mutation in most cases, giving the polar effect. It seems likely from the evidence of Malamy (1966) with mutants of the lac operon and Imamoto et al.(1966) in the trp operon that the ribosomes remain on the mRNA after translation has stopped at a nonsense codon, and some 'persevere' along the mRNA until reaching the next initiating codon, while the remainder fall off in this non-translating phase. Since the degree of polarity has been shown to depend on the distance of the nonsense codon from the end of the cistron (i.e. from the next initiation codon)(Newton et al., 1965; Newton, 1966), an insertion of bases operator distal to the nonsense mutation would

increase this relevant distance - hence the extreme polarity of the insertion mutations.

In our ochre mutant NA 121 we find, as in the case of NA 156, Gal⁻3 and Gal⁻9, that there is more RNA in the cell than active protein translated from it. Thus this situation appears to be consistent with the presence of a nonsense codon.

Morse and Yanofsky (1969) and Morse and Primakoff (1970) have shown by kinetic hybridization studies that mRNA from nonsense mutants of the trp operon is degraded more quickly than that from wild type cells, and suggest that this is due to premature exposure to nuclease activity. This breakdown in the trp operon has been shown to start from the 5' i.e. operator proximal end of the molecule (Morse et al., 1969; Morikawa and Imamoto, 1969). It is well established that the rapid decay of mRNA is prevented by the attachment of ribosomes to it. The presence of the 30S sub-unit is essential (Forchhammer and Kjeldgaard, 1968) although actual translation is not necessary since inhibition of peptide bond formation during protein synthesis without inhibition of ribosome movement by amino acid starvation (Forchhammer and Kjeldgaard, 1968; Lindahl and Forchhammer, 1969), by addition of low levels of chloramphenicol (Levinthal et al., 1963; Medhi and Yudkin, 1967, 1968; Contesse and Gros, 1968; Lindahl and Forchhammer, 1969) or by anaerobic growth conditions (Levinthal et al., 1963) all result in the protection of mRNA. The translation of the mRNA before a nonsense

codon and the presence of some ribosomes along the non-translated region immediately following this would thus protect this mRNA from premature breakdown, and could conceivably also delay nuclease attack on the ribosome-free distal RNA from the 5' end.

If this is true then, presumably, some of the RNA from the ochre mutant NA 121, which we pick up in the hybrid is not being translated beyond the nonsense codon. This RNA could either be in the process of breakdown from the 5' end but still large enough to be collected in our preparation or could be complete due to the protection of the ribosome-free distal RNA by the ribosome-associated operator end of the molecule. A similar situation would arise in polar mutants which were due to a nonsense mutation in an insertion.

If the polarity of our mutants NA 156, Gal^-3 and Gal^-9 were due to an insertion carrying a nonsense codon the polarity would be relieved without concommitant return to Gal^+ phenotype when the mutations were examined in their specific suppressor positive background. This does not happen when ochre and amber suppressors are used (Bishop, unpublished work). No increase in kinase or transferase enzymes activities was found in the presence of the ochre and amber suppressors used. The effect of a UGA suppressor in this context has not been tested.

Finally, these mutations could be of the frameshift type produced by the inserted region of DNA. This could result in normal

transcription and translation with the synthesis of inactive proteins or the frameshift could give rise to a nonsense codon further along the cistron. If the former occurred one would expect normal wild type levels of mRNA should be found. From the value of F_G for Gal$^-$9 (1.59) and allowing for experimental error it is possible that Gal$^-$9 may fall into this category. The results for NA 156 and Gal$^-$3 do not fit this theory but could be the result of a nonsense codon generated by the frameshift. If this were the case, then in the presence of the appropriate su$^+$ allele normal levels of mRNA would be transcribed while inactive protein would be synthesised due to the frameshift. This has not been tested.

In order to test the hypothesis of a frameshift caused by an insertion two procedures could be carried out. Both of these test for relief of polarity without the actual suppression of the initial mutation.

ICR compounds were used in an attempt to revert the polar mutations without success. By using these mutagens and examining the treated cells for kinase and transferase activities one could determine whether or not polarity is relieved. Increase in enzyme activities after treatment would suggest that the mutation contained a frameshift, without distinguishing between this producing inactive protein or generating a nonsense codon. The second means of testing this hypothesis would utilise the "su-A" suppressor of Beckwith (1963). This "su-A" suppressor has been shown to relieve polarity in nonsense

mutants of the lac operon (Beckwith, 1963; Scaife and Beckwith, 1966), and in nonsense and frameshift mutants of the trp operon (Morse and Primakoff, 1970) as measured by mRNA levels, without suppression of the original mutation. Morse and Primakoff (1970) demonstrated that this was probably due to the "su-A" mutation resulting in a defective nuclease which broke down mRNA at a slower rate than the wild-type enzyme. Increase in mRNA levels in the presence of the "su-A" gene would suggest that a frameshift mutation had generated a nonsense codon.

VI. SUMMARY

1. Hybridization saturation and competition experiments were carried out using λ dg DNA and RNA from D-fucose induced and TMG repressed E.coli K12. A method of analysis of the data is described and was used to calculate the relative levels of gal mRNA in different RNA preparations. The relative enzyme activities and mRNA levels in induced and repressed cells were compared. The significance of the results is discussed in the context of the present state of our knowledge of the interaction of transcription and translation and of operon control.

2. A basic genetic analysis of five polar mutants of the epimerase gene of the gal operon of E.coli K12 and their revertants was carried out. The level of gal mRNA in these mutants compared to wild type was measured using hybridisation competition experiments and the method of analysis described. The results obtained are discussed in relation to current theories on polarity.

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