ABSTRACT OF THESIS

Name of Candidate MICHAEL GOMAN

Address Department of Molecular Biology, King's Buildings

Degree Master of Science *Date* September 1973

Title of Thesis Mutations affecting regulation of the lac operon of Escherichia coli.

In the introduction I have discussed the lac operon and its control region. Special attention has been paid to the control of the transcription process. Also briefly discussed are the arabinose operon, RNA polymerase, and factors involved in the intiation and termination of transcription.

The experimental studies describe the isolation of temperature sensitive mutants affecting RNA synthesis. The alt-1 mutation is shown to make the synthesis of a major class of unstable RNA temperature-sensitive. Temperature-sensitivity is also conferred on the synthesis of β-galactosidase and arabinose-isomerase. This temperature-sensitivity is probably due to a lesion(s) in a single gene which maps in the small region of the chromosome covered by the KLF2 episome. The alt-1 protein appears to be directly involved in the transcription process and may be able to distinguish between different classes of promoters.

MUTATIONS AFFECTING REGULATION OF THE LAC OPERON OF *ESCHERICHIA COLI*

by

Michael Goman, B.Sc. (Surrey)

A thesis presented for the degree of Master of Science

M.R.C. Molecular Genetics Unit
Department of Molecular Biology
University of Edinburgh

September, 1973

ABSTRACT

In the introduction I have discussed the *lac* operon and its control region. Special attention has been paid to the control of the transcription process. Also briefly discussed are the arabinose operon, RNA polymerase, and factors involved in the initiation and termination of transcription.

The experimental studies describe the isolation of temperature sensitive mutants affecting RNA synthesis. The *alt-1* mutation is shown to make the synthesis of a major class of unstable RNA temperature-sensitive. Temperature-sensitivity is also conferred on the synthesis of β-galactosidase and arabinose-isomerase. This temperature-sensitivity is probably due to a lesion(s) in a single gene which maps in the small region of the chromosome covered by the KLF2 episome. The *alt-1* protein appears to be directly involved in the transcription process and may be able to distinguish between different classes of promoters.

LIST OF CONTENTS

PART 1 - INTRODUCTION

PART I

INTRODUCTION

I. The structural genes of the lac operon

There are two proteins directly concerned with lactose catabolism, β-galactosidase (β-gz) and lactose permease (M protein). There is also a third gene in the operon coding for thiogalactoside transacetylase.

a. β-galactosidase (β-gz)

The gene coding β-gz monomer is known as the z gene. It was shown decisively to code for β-gz by Cohn and Torriani (1952) who found that missense z^- mutations which did not have any β-gz activity still produced material which reacted with β-gz antibody. Since then a Lac^- mutant has also been described from which β-gz activity could be recovered following addition of β-gz antibody and this activity was proportional to the amount of antibody added (Rotman, Celada, 1968).

β-gz is a tetramer made up of four identical subunits each of 135,000 daltons (Craven, Anfinsen, Steers, 1965). The active tetramer cleaves lactose at the 1,4, linkage to yield glucose and galactose. It also changes the 1,4 linkage to a 1,6, linkage to give allolactose the real inducer of the lac operon (Jobe, Bourgeois, 1972a).

b. Lactose permease

Early studies on the lactose system revealed a class of mutants which, while they still produced wild type levels of β-gz in response to a gratuitous inducer isopropylthio-β-D-galactoside (IPTG),

were still phenotypically Lac$^-$. These mutants were shown to be due to a defect in an inducible permease which mapped close to the z gene in Hfr crosses (Rickenberg, Cohen, Buttin, Monod, 1956).

The lactose permease or 'M' (membrane associated) protein was shown to be the product of the y gene in several ways. β-D-galactosyl-1-thio-β-D-galactoside (TDG) (which has a specific affinity for 'M' protein) binding activity could not be detected in y$^-$ cells (Rickenberg et al, 1956). A class of mutants, not z$^-$, was obtained which were unable to hydrolyse orthonitrophenyl-β-D-galactoside (ONPG a substrate for β-gz) at high temperature (42^{o}C). This property was explained by the discovery that at 42^{o}C they were unable to accumulate many galactosides. Furthermore, in vitro studies showed that these strains have lesions which map close to the z gene and produce a protein whose binding of TDG is temperature-sensitive, (Fox, Carter, Kennedy, 1967).

Finally Jones and Kennedy (1969) showed that strains haploid, diploid and triploid for the y gene bound proportional amounts of TDG and also that proportional amounts of TDG binding 'M' protein could be isolated from the membrane.

The advantages of having a specific inducible transport protein for concentrating metabolites inside a cell are obvious. It is much more efficient to produce such proteins only when they are specifically needed, but it does suggest a paradox. If there is no transport protein present before induction, how can the metabolite be carried into the cell to activate induction? There are at least two possible models which resolve this paradox. Firstly the transport protein could be produced constitutively at a low level so that there

is always enough transport of the metabolite to permit induction. The uninduced level of 'M' protein must be very low as the uninduced level of β-gx, the first gene in the operon, is 1/1000 of the fully induced level. Whether this level is able to transport enough lactose to permit induction is not known. Secondly, there could be a generalised transport system which works with low efficiency but carries enough metabolite into the cell to permit induction. This may be a function of the phosphotransferase system, (Kundig, Ghosh, Roseman, 1964) a possibility which is supported by its effects on inducer exclusion (See 1, V, a)

The molecular weight of the lactose permease has been estimated as 31,000 daltons (Jones, Kennedy, 1969) and 29,000 daltons (Guthrie, Pardee, 1969) but as many proteins dissociate into subunits on sodium dodecyl subphate treatment this may only be the weight of a subunit of the functional molecule.

The discovery of a second transport system for melibiose which was distinct from the lac system (Prastidge, Pardee, 1965) led to the development of an easy method for distinguishing between z^- and y^- mutations. The melibiose transport system is temperature-sensitive in E. coli K12 and so does not function at 42°C. Both z^- and y^- are Lac^- but the z^- (unless it is strongly polar) can still accumulate lactose and or melibiose. The z^- strain can grow on melibiose at both 30°C and 42°C as it can accumulate melibiose at 42°C using the y gene product. The y^- strain can grow on melibiose at 30°C but not at 42°C since at 42°C it will have no active melibiose permease or lactose permease.

c. Thiogalactoside transacetylase

The third structural gene of the lac operon, the a gene, codes for the enzyme thiogalactoside transacetylase. No function for this enzyme in lactose metabolism has been found nor is it involved in the accumulation of galactosides, (Fox, 1966). Despite the lack of obvious function for this enzyme it has proved very useful in the study of catabolite repression and polarity effects in the lac system.

Definite proof that the a gene codes for transacetylase was obtained with the isolation of deletions which removed a but not z or y. Zabin, Kepes and Monod,(1962) also showed that the product of a gene which mapped close to y was inducible with IPTG and could acetylate a number of substrates.

II. The Operon model and its control elements

The operon model was first proposed by Jacob and Monod (1961) to explain co-ordinate induction of β-gz and transacetylase. Poor inducers allowed only low levels of both enzymes while with good inducers high levels of both enzymes were obtained. Jacob and Monod (1965) on evidence from Hfr crosses and Fox (1966) from deletion analysis proposed that the structural genes were contiguous and transcribed as a single unit in the order z y a. (Jacob, Monod 1961)

At the same time as they proposed the operon model Jacob and Monod also predicted the need for a repressor molecule to control the expression of the structural genes of an operon. How this repressor is made and functions has been the subject of intensive study which is dealt with in sections (1,II,b,c,d; 1,III,a,b,c,). At the moment I will just give the current status of the lac operon and its control

region

i is the gene coding for the repressor, *p* is the promoter region, *o* the operator region, *z y a* are the structural genes.

a. The regulatory region

The regulatory region is responsible for controlling the expression of the structural genes. It is so far known to have three parts, the *i* gene which codes for the repressor, the promoter region which interacts with RNA polymerase holoenzyme (1,VII,a) and other factors (1,VII,g) and the operator region which interacts with the repressor (1,IIIa,c).

b. The i gene

The *i* gene has its own promoter which presumably works with low efficiency. Repression is only slowly established on the introduction of an i^+ gene into an i^- background, which is indicative of slow repressor synthesis, (Pardee, Jacob, Monod, 1959) giving 5-10 repressor molecules per gene copy (Muller-Hill, Crapo, Gilbert, 1968). There is a transcriptional stop signal at the end of the *i* gene which is read in the same direction as the structural genes (Miller, Reznikoft, Ippen, Signer, Beckwith, 1970) (see section 1,II,c). Strains producing a temperature-sensitive repressor have been isolated (Horiuchi, Yasami, 1966) and suppressible amber i^- strains have also been isolated (Bourgeois, 1965; Muller-Hill, 1966). The repressor is almost certainly completely protein and contains little, if any,

nucleic acid (Riggs, Suzuki, Bourgeois, 1970).

The repressor acts in trans and must therefore be cytoplasmic as is shown in diploid studies in that an i^+/i^- strain is Lac$^-$ inducible. (Pardee et al, 1959).

c. Mutations affecting the i gene and its expression

In addition to the i^- mutations discussed above the i gene can yield other mutations of crucial importance to our understanding of repressor action.

i^S

Two non inducible mutations termed i^S for super repressor have been described (Wilson et al, 1964). They code for a repressor with a much reduced affinity for IPTG but the same affinity for operator DNA (Bourgeois, Jobe, 1970). Repressor DNA interactions are discussed in section 1,III,a,c).

i^{-D}

Mutants have been isolated which are constitutively Lac$^+$ even in a diploid strain with an i^+ in trans. These mutations all map near the N terminal end of the i gene (Pfahl, 1972) and have been called i^{-D} as they are dominant to i^+. These properties are discussed in section 1,II,d.

i^Q, i^{SQ}

A strain which produces ten times more repressor than wild type has been isolated and called i^Q (Muller-Hill et al, 1968), and Miller (The lac operon, 1970) has isolated an i^{SQ} which makes fifty times more repressor than wild type. These two mutations probably

affect the *i* gene promoter permitting more efficient initation of transcription.

Strains containing the i^Q mutation have been used to establish that the *i* gene is read in the same direction as the structural genes. A strain containing both i^Q and *Li* (which deletes the transcriptional stop signal at the end of the i gene and so allows read through from the *i* gene) produces more β-gz than a strain containing *Li* alone (Miller, Muller-Hill, Beckwith, 1968).

i^{X86}

Jobe and Bourgeois (1972) have isolated a strain which produces a repressor which has a forty-fold greater affinity for lac operator DNA. This repressor could prove useful in sequencing operator DNA.

d. *i subunit interactions*

The dominance effect of the i^{-D} mutations can be explained by invoking the tetrameric structure of the functional repressor. In an i^+/i^{-D} diploid strain most of the repressor tetramers will contain at least two i^{-D} subunits which will lower the affinity of the whole tetramer for operator DNA and so the strain will remain i^-. This model predicts that an i^Q/i^{-D} strain, in which there are ten i^+ subunits, will have tetramers mainly composed of i^+ subunits and will be i^+. Similarly, an i^S/i^+ diploid should be uninducible due to each tetramer containing at least two i^S subunits but an i^Q/i^S diploid will be inducible. These strains have been constructed by Muller-Hill, Crapo and Gilbert (1968) and give the predicted results.

e. The promoter region

The promoter is that part of the DNA of the control region to which RNA polymerase holoenzyme binds to give correct initiation of transcription. The nature of this binding predicts two classes of promoter mutations. One produces a reduction in transcription by inhibiting binding and one increases transcription by increasing RNA polymerase binding.

f. Promoter mutation which reduce expression of the operon

Mutations of this class were isolated by Scaife and Beckwith (1966) and called L1, L8 and L37. Mapping of these mutations against supposed deletions which stretched from the operator into the i gene placed the promoter between the operator and z. Later work showed these supposed deletions to be double o^c i^- point mutations and so this placing of the promoter could be wrong. Mapping of these promoter mutations against deletions coming into the regulatory region from the structural genes placed the promoter between the operator and the i gene (Miller, Ippen, Scaife, Beckwith, 1968) (Ippen, Miller, Scaife, Beckwith, 1968).

The L1 promoter mutation is a small deletion which removes part of the promoter and the end of the i gene. L8 and L37 are point mutations unable to recombine with L1. L1 is only partially inducible, the uninduced level being 60% of the induced level which is 2% of wild type induced level. This low induction ratio is thought to be due to the production of a faulty repressor. L8 and L37 are inducible and produce 6% of wild type levels of β-gz when induced.

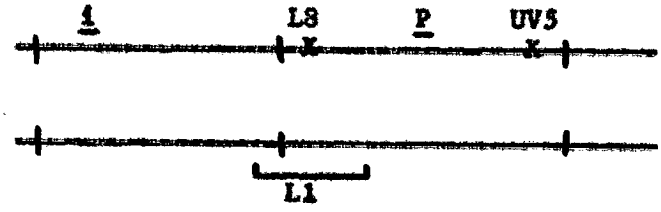
g. Up promoter mutations

An up promoter mutation has been isolated (Silverstone, Arditti, Magasanik, 1970) by selecting Lac$^+$ revertants of L8 after UV mutagenesis. It is called UV5 and has 50% of wild type β-gz activity in glycerol grown cultures. UV5 can be separated from L8, by P1 transduction, at a very low frequency which suggests that they are very closely linked. β-gz synthesis in the UV5 strain is insensitive to catabolite repression (section I,V,b) but this characteristic is due to the original L8 mutation.

A mutation which increases the expression of the lac operon both in vivo and in vitro has been described (Chen et al, 1971) and called p^S. It is not clear whether this is due to an alteration in the existing promoter or to the insertion of an entirely new promoter between o and z.

h. Genetic evidence for two regions in the promoter.

It has been proposed that the promoter comprises two regions (Beckwith, Grodzicker, Arditti, 1972): one which is operator distal and interacts with the crp (a protein involved with the transcription of catabolite sensitive genes) c-AMP (3'5' cyclic adenosine monophosphoric acid) complex and another which is operator proximal and interacts with RNA polymerase holoenzyme. This conclusion is drawn from genetic experiments in which mutations in the two postulated regions are separated by recombination.



It can be seen from the above diagram that a recombinant containing L1 and UV5 can only be obtained if UV5 maps outside the region deleted by L1 and so outside the region interacting with the crp-c-AMP complex.

i. The Blattner and Dahlberg promoter model

The possibility of a more complex system for promoter function has been raised by work on the phage λ by Blattner and Dahlberg (1972) who discovered that there is an untranscribed region of about 200 base pairs intervening between the RNA polymerase binding site and the base pair at which transcription begins. The operator appears not to be transcribed. This type of model cannot be applied directly to the lac operon since the lac operator is transcribed (section 1,II, e).

The convention of representing DNA as a linear molecule tends to prejudice thinking about the structure of localised regions of DNA. It is perfectly possible that the lac promoter region is folded so that regions which on the linear map appear to be distinct are in fact closely associated. Blattner and Dahlberg (1972) raised this possibility for λ. Folding of the DNA may bring the RNA polymerase binding site very close to the transcriptional start site so eliminating the drift region as a physical entity.

j. The operator

The operator can be defined in genetic terms as that part of the control region which can yield constitutive mutants which do not inactivate the repressor. Operator constitutive (O^c) mutants bind repressor less efficiently than wild type operators (Smith, Seidler, 1971). The operator is DNA (Gilbert, 1967) φ80 dlac DNA binds repressor φ80 wild type DNA does not, repressor bound to φ80 dlac DNA

can be removed with IPTG.

k. Operator constitutive mutations

Operator constitutive mutants can be divided into two classes by the degree to which they are constitutive. Sadler and Smith (1971) have proposed on genetic evidence that each class of constitutive mutation can be said to be doubly degenerate, that is that each class can be caused by a mutation at one of two points which are placed symmetrically with respect to the centre of the operator. This suggests that the operator is composed of two halves which are mirror images. This model has been confirmed (Gilbert - personal communication) who has sequenced the lac operator and shown it to have a two-fold symmetry).

1. The operator sequence

The method used to sequence the operator DNA is to transcribe isolated operator DNA into RNA and to sequence the transcript. An O^c mutant has also been sequenced and shows a base pair changed from a GC to an AT in one of the symmetrical regions.

The technique used in these sequencing studies should make it possible to elucidate the mechanism of repressor action and also to sequence other parts of the control region. This should help us to understand the nature of the promoter interactions with RNA polymerase and the crp-cAMP complex.

m. The operator is distinct from the z gene

Bhorjee, Fowler and Zabin (1969) have shown biochemically and Reznikoff and Beckwith (1969) genetically that the operator is

distinct from the z gene. β-gz made in a trp lac fusion strain in which part of the operator is removed has the same physical properties as wild type β-gz and it also has the same threonylmethionine N terminal sequence. In this strain which is under the control of the trp promoter and is not repressed by the lac repressor active β-gz and transacetylase are made.

Gilbert has shown using UV5 DNA that the operator is transcribed but it is probably not translated as no amber operator mutations have been isolated (Bourgeois, 1965) nor do partial deletions of the operator alter the physical properties of β-gz (Bhorjee et al, 1969).

III. The lac repressor and its interactions with DNA and inducers

In vitro studies on repressor DNA interactions were not possible, because of its very low concentration in the cell (0.002% per genome of the total protein), until the development of the IPTG equilibrium dialysis technique (Gilbert, Muller-Hill, 1966). This technique depends on the binding of radioactive IPTG to repressor in a dialysis sac but as the amount of repressor in wild type cells is very low a blind partial purification is necessary. To improve the chance of detecting IPTG binding a mutant called i^t was isolated, it was isolated as being inducible by a low concentration of IPTG and binds IPTG more tightly than wild type repressor. In the assay an aliquot of an ammonium sulphate fraction is dialysed against a solution of radioactive IPTG and a fraction which binds IPTG is detected in the dialysis sac. This material was identified as a protein. It could not be detected in i^-am or i^s cell extracts, (i^s repressor binds IPTG

less firmly than wild type repressor (Jobe, Riggs, Bourgeois, 1971).

With the isolation of the i^{Q} and i^{SQ} mutations it is now possible to isolate larger quantities of repressor. When the i^{SQ} mutation is put onto a phage it is possible to get a cell to produce one thousand times more repressor than wild type cells, (2.5% of the total protein) and a yield of several grammes per kilogram of cells is possible. Riggs and co-workers (Riggs, Bourgeois, 1968)(Riggs, Bourgeois, Newby, Cohn, 1968) have developed the repressor isolation technique, by precipitating the repressor with antibody, and obtain repressor which is much purer than the Gilbert, Muller-Hill preparation.

a. Repressor DNA binding

In vitro assays using nitrocellulose filter binding (Riggs *et al*, 1968) (Riggs, Suzuki, Bourgeois, 1969) have shown the association constant of repressor and operator to be very fast and the dissociation constant to be slow which gives a repressor operator complex half life of about 30 minutes. The binding is dependent on the presence of Mg^{++} ions. Experiments using dilute solutions of repressor and DNA suggest that the time taken for the repressor to find the operator DNA by diffusion is not rate limiting and it may be possible that the repressor binds to the phosphate backbone of the DNA and then travels along the DNA until it finds the operator where it binds firmly (Lin Riggs, 1972). This model is possible as experiments show that if Mg^{++} ions are omitted from the reaction mixture general binding of repressor to DNA masks the binding repressor to operator DNA (Gilbert, Muller-Hill, 1970).

b. Repressor inducer interactions, i^+, i^s

Inducers of wild type i^+ strains can be placed in an unambiguous order of efficiency from IPTG the best, to melibiose the worst. Bourgeois and Jobe (1970) have isolated a series of i^s strains which can be arranged without exception in an hierarchy in that any one that is not induced by a galactoside high on the list can never be induced by a galactoside lower on the list. This rigid order relating all the i^s strains to the efficiency of the inducer shows that the repressor recognizes the inducer. They also showed that except in one case the repressor operator binding and half life were the same as wild type suggesting that generally the repressor of i^s mutations does not have an altered affinity for operator DNA. IPTG not only interacts with free repressor but also interacts with repressor operator complexes, reducing the half life of these complexes (Rigg, Bourgeois, 1969). In general the i^s mutations show a similar response to IPTG when bound to operator DNA but a higher concentration of IPTG is needed to promote dissociation of repressor operator complexes.

c. The i^{X86} repressor, its interactions with DNA and inducers

The i^{X86} repressor producing strain shows an unusual induction pattern. It is partially constitutive showing 2% of induced wild type activity. The constitutive activity is repressed by low levels of IPTG but is induced by high concentrations of IPTG to a maximum level of 2% of wild type activity. A possible explanation for this induction pattern invokes the tetrameric structure of the repressor molecule. The repressor subunits of the i^{X86} gene could be distorted in such a way that it has a reduced affinity for DNA with a resultant

low level of constitutivety of the lac operon. Addition of low levels of IPTG could restore some of the subunits to their functional conformation with a concurrent effect on the other subunits in the tetramer giving a tetramer which has one or two IPTG molecules bound to it and is fully active as a repressor. Addition of a higher concentration of IPTG could give a low level of induction as one would expect from a repressor with an increased affinity for operator DNA.

d. Lactose is an anti-inducer

Jobe and Bourgeois (1973) have shown that lactose galactose and ONPF (ortho nitrophenyl-β-D-fucoside) are anti-inducers of the lac operon. The anti-induction of the lac operon by lactose is not due to catabolite repression by the glucose derived from the splitting of lactose nor by inducer exclusion (section I,V,a). Lactose acts on the repressor operator complex slowing down the dissociation of the complex five-fold. It also competes with the inducers allolactose and IPTG for the repressor operator complex thereby reducing the efficiency with which the inducer can remove the repressor from the operator.

IV. Repressor Binding and Transcription

Two models have been proposed for repressor action. The bound repressor prevents polymerase from binding to the promotor or bound repressor prevents bound polymerase transcribing. Which of these models is correct is still not clear as Eron and Block (1971) say that bound repressor prevents binding of the polymerase and or the crp-cAMP complex. Chen et al (1971) say that they can get

rifampicin resistant complexes in the presence of repressor which means that the polymerase must be bound to the DNA.

V. The Glucose Effect

The inhibition of β-galactosidase formation by glucose was first observed by Monod (1947). Glucose has since been found to affect the expression of many other catabolic enzymes: - lac permease, galactokinase, glycerokinase, L α-glycerophosphate permease, enzyme II for fructose of the phosphotransferase system, L-arabinose permease, tryptophanase, D-serine deaminase and thymidine phosphorylase (de Crombrugghe et al, 1969) and probably generally affects the synthesis of catabolic enzymes.

Experiments by Epstein, Naono and Gros (1966) showed that when E. coli was grown in a mixture of glucose (0.4 mg/ml) and lactose (2 mg/ml) no β-galactosidase was synthesised until the supply of glucose was exhausted. There was then a lag period in growth during which β-galactosidase was synthesised very rapidly after which growth resumed at a slower rate with a parallel production of β-galactosidase.

a. Inducer exclusion

Clark and Marr (1964) attributed the glucose effect to a glucose-induced increase in the amount or activity of the lac repressor thereby blocking the production of lac permease. There is no direct evidence to support Clark and Marr's proposition. Cohn and Horibata (1959) have shown that glucose has a little effect on lac inducer (methyl-β-D-thiogalactoside TMG) uptake by the lac permease. However, Kepes (1960) has shown that glucose strongly inhibits uptake not mediated by the lac permease. Thus the glucose effect could, at

least in part, be attributed to its effect on a permeation system independent of the y gene product. Pastan and Perlman (1969) and Kabak (1969) have observed that glucose-1-phosphate, which is a product of the phosphotransferase system (Kundig, Ghosh, Roseman, 1964) inhibits the uptake of galactose, fructose and lactose suggesting that this system may be involved in primary lactose uptake.

The most likely explanation for inducer exclusion is that it acts at the level of the phosphotransferase system and that this system is used for only a brief period in lactose transport. This glucose sensitive system may serve to prime lac operon induction, giving a level of lac permease sufficient to provide full induction of the y gene permease.

b. Transient and catabolite repression

When cells grown in glycerol and the presence of an inducer not subject to exclusion (IPTG) are exposed to 10 mM glucose, there is a complete cessation of β-gz synthesis for up to half a generation (Moses, Provost, 1966), the overall growth rate during this period is slightly increased so the effect is not due any overall inhibition of protein synthesis, this phenomenon is known as transient repression. After the period of transient repression β-gz synthesis restarts but at a rate lower than the pre-glucose rate, this is attributed to catabolite repression. Makman and Sutherland (1965) found that shortly after the addition of glucose to glycerol grown cells there is a rapid drop in the intracellular level of cAMP and that during catabolite repression the cAMP level is lower than in glycerol grown cells. Exogenous addition of cAMP reverses both transient and catabolite re-

duced so that it can respond rapidly to environmental changes. The lac system shows all these characteristics, it is economical in that only one protein produced at a low level is needed for shut off of the entire operon, it is efficient in that the basal level of enzyme production is only 1/1000 of fully induced level, and inducer response is rapid in that maximal rate of enzyme synthesis is reached in a few minutes. The basal level of enzyme synthesis is probably necessary for rapid induction as the real inducer is allolactose, therefore a low level of β-galactosidase in the cell, at all times, is required to convert the substrate lactose to the inducer allolactose before maximum induction can occur.

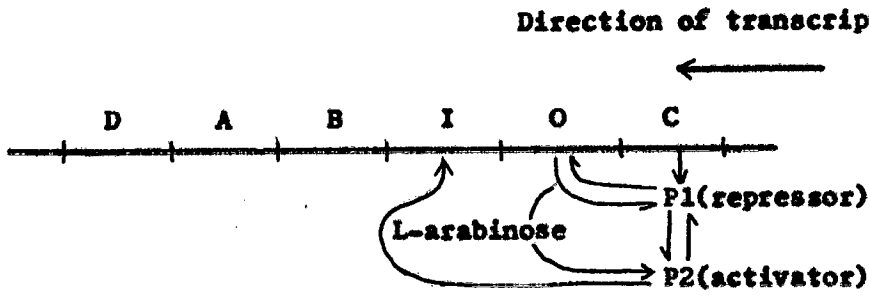
The need for a second control system

The negative control system while being very efficient does not allow for the presence, at the same time as lactose, of a less complex substrate such as glucose. The control of synthesis of the lac enzymes in the presence of substrates such as glucose is controlled by two other systems, inducer exclusion and catabolite repression (sections 1,V,a and 1,V,b). Control by catabolite repression is by the crp-cAMP system which is a positive control system.

VI. The Arabinose Operon

Control of expression of the *ara* operon is in some ways similar to the *lac* system. It has a repressor which binds to the operator but the mechanism of induction is different. The crp-cAMP complex is required for maximum expression of the operon. The operon is susceptible to transient and catabolite repression which are relieved by cAMP.

The main difference between the *lac* and *ara* control systems is the dual functions of the *ara C* protein as proposed by Engelsberg, Sheppard, Squires, Meronk, (1969). Below is a diagrammatic representation of the scheme they proposed.



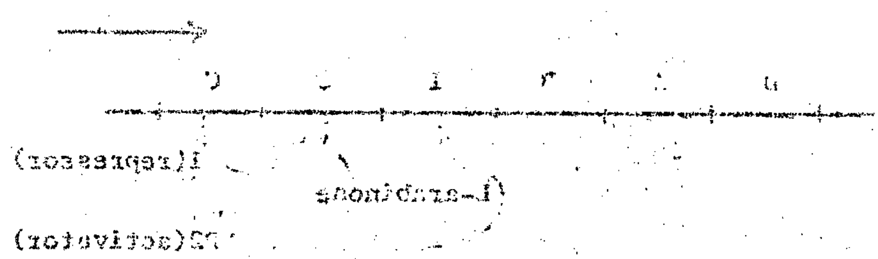
D, *A* and *B* are the structural genes.
I is the site of initiation of transcription which binds the polymerase, crp-cAMP complex and the activator P2. *O* is the operator which binds the repressor P1 and is analogous to the *lac* operator. *C* is the gene coding for the *ara C* protein in a limited way analogous to the *i* gene of *lac*.

Isolation of *ara* C^- mutations of both deletion (Sheppard, Engelsberg, 1967) and nonsense classes (Irr, Engelsberg, 1967) which were negative for the expression of all the structural genes, and therefore necessary for gene expression, led to the proposition that

VI. The Arabinose Operon

Control of expression of the ara operon is in some ways similar to the lac system. It has a repressor which binds to the operator but the mechanism of induction is different. The crp-cAMP complex is required for maximum expression of the operon. The operon is susceptible to transient and catabolite repression which are relieved by cAMP.

The main difference between the lac and ara control systems is the dual functions of the ara C protein as proposed by Englesberg, Sheppard, Squires, Meronk, (1969). Below is a diagrammatic representation of the scheme they proposed.



D, A and B are the structural genes.

I is the site of initiation of transcription which binds the polymerase, crp-cAMP complex and the activator P2. O is the operator which binds the repressor P1 and is analogous to the lac operator. C is the gene coding for the ara C protein in a limited way analogous to the i gene of lac.

Isolation of ara C^- mutations of both deletion (Sheppard, Englesberg, 1967) and nonsense classes (Irr, Englesberg, 1967) which were negative for the expression of all the structural genes, and therefore necessary for gene expression, led to the proposition that

the ara C protein has a positive control function. This makes it quite unlike the lac repressor which has only a negative control function so that i^- strains are constitutively Lac^+. While the ara C gene product has a positive control function it also has a negative control function in which it binds to the operator and prevents transcription. Engelsberg, Squires, Meronk, (1969) have demonstrated in diploid studies that an ara C^+ gene trans to a deletion of ara C, that does not remove a site which they postulate is an operator, stimulates arabinose isomerase production two-fold. An ara C^+ trans to an ara C deletion encompassing the postulated site stimulates arabinose isomerase synthesis thirty-five-fold. As these experiments were performed in the absence of inducers they show that the ara C protein has a repressor activity as well as a stimulatory activity. These experiments also show the order of the control region to be I O C in that it is possible to delete O with a deletion running in from the C gene and without affecting I.

It also seems probable that for complete induction of the arabinose operon the absence of the C gene product in its repressor form (P1) is necessary. Mutations in the ara C gene (called C^c) have been isolated in which expression of the operon is constitutive, that is independent of inducer (Engelsberg, Irr, Power, Lee, 1965). In a diploid strain C^+/C^c, C^+ is dominant in uninduced cultures suggesting that binding of the C^+ gene product inhibits expression of the ara genes under the positive control of the C^c gene product. It is formally possible that this effect is due to subunit mixing of the i^s/i^+ type in the lac system but the model in which P1 bound to the operator inhibits the action of the activator is more attractive.

Induction occurs in the arabinose system by the inducer shifting the equilibrium between P1 and P2 largely towards the P2 activator form.

RNA polymerase and Transcription Factors

The transfer of information from DNA into protein involves two major processes, transcription and translation. Transcription is the process by which RNA molecules which are an exact copy of the DNA base sequence is made, they can be copies of single genes or entire operons. Translation is the process by which the information in the RNA transcript is converted into the amino acid sequence of the protein. Transcription is catalysed by an enzyme DNA dependent RNA polymerase.

a. Structure of RNA polymerase

Highly purified RNA polymerase contains several different polypeptides named, β', β, α_2, σ and W. The W subunit is not required for *in vitro* RNA synthesis and has no known function. The enzyme in this form is called holoenzyme, it can dissociate reversibly into core ($\beta'\beta\alpha 2$) and sigma (σ).

b. Sigma factor

Sigma factor is not necessary for transcription as core will transcribe calf thymus DNA (Burgess, Travers, Dunn, Bautz, 1969) and poly dAT (Berg, Barrett, Hinkle, McGrath, Chamberlain, 1969). Sigma factor is necessary for correct initiation of transcription at promoter sites to which holoenzyme binds more tightly than does core to DNA in general (Losick, 1972). Sigma factor is required only for correct initiations, after initiation it is released from the enzyme DNA complex, and can be reused, leaving the core enzyme to extend initiated RNA chains (Travers, Burgess, 1970) (Stonington, Pettijohn, 1971).

How sigma confers this specificity for promoter sites on the

holoenzyme is not known. One model suggests that sigma itself contains information necessary for promoter recognition either as itself or as part of the holoenzyme or by altering the core enzyme so that it recognises specific base sequences in the DNA. Another model suggests that core enzyme itself contains the information for promoter recognition and that sigma prevents non promoter binding thereby enhancing the chance of the core enzyme finding a promoter. The answer to this question could best be gained by isolation of other sigma factors (if they exist) which confer on the core enzyme specificity. for promoters other than those specified by sigma.

c. The β subunit

Mutations have been isolated which confer resistance to the drug rifampicin, these mutations have been shown to be in the β subunit (Heil, Zillig, 1970). When rifampicin sensitive holoenzyme and DNA are allowed to form a complex in the absence of nucleoside triphosphates (pre-initiation complex) this complex will make RNA chains if rifampicin and nucleoside triphosphates are added together. The β subunit is therefore probably involved in the formation of the DNA holoenzyme pre-initiation complex.

d. The β′ subunit

The β′ subunit has been implicated in the primary binding of holoenzyme to DNA as heparin, which competes for DNA binding with enzyme and can displace it from DNA, has been shown to bind exclusively to the β′ subunit (Walter, Zillig, Palm, Fuchs, 1967).

e. The α subunit

The α subunit is essential for RNA synthesis but no specific properties for it in the transcription process have been identified.

Factors Stimulating Transcription

a. The ψr Factor

The ψr factor (Travers, Kamen, Schleif, 1970) was originally thought to differentially stimulate the synthesis of ribosomal RNA (rRNA) but it could not be considered as another sigma like factor as it needed sigma for its stimulatory activity. The picture concerning ψr has become blurred since Haseltine (1972) and Pettijohn (1972) have shown ψr to have a general stimulatory effect on RNA synthesis and can find no preferential stimulation of rRNA synthesis.

b. The crp factor

The role of the crp factor in the transcription of catabolite sensitive genes has been discussed earlier. Suffice it to say here that it is necessary along with c-AMP for the maximal *in vitro* transcription of all catabolite sensitive genes tested and acts at a site which can be genetically separated from the holoenzyme binding site in the lac promoter (Beckwith, Grodzicker, Arditti, 1972).

c. The ara C protein

The *ara C* protein has a dual function, it acts as both repressor and activator of transcription (1.VI).

Using an *in vitro* system, Lang-Yang and Zubay (1973) have shown that added *ara C* protein can circumvent the need for the crp cAMP complex for *ara* gene expression. The *ara C* protein acts, in its

activator form, at the site of initiation of transcription and it is therefore possible that it could directly substitute for the crp cAMP complex.

d. The M Factor

A protein has been partially purified from E. coli cells (Davidson, et al, 1969) which stimulates transcription from T4, T7, E. coli DNA's but particularly from λ DNA. It is not a substitute for sigma as it can stimulate holoenzyme saturated with sigma and does not stimulate core enzyme. M factor appears to stimulate transcription by interacting with polymerase either by binding to it or modifying it in some way as enzyme mixed with M factor and then recovered by centrifugation retains its enhanced activity (Davidson et al, 1970).

e. Other factors

Several other factors have been described but as they are mainly concerned with phage transcription they need not be discussed here.

VIII Termination of Transcription

The presence of transcriptional termination signals has been demonstrated using deletion strains such as L1 and fusion strains such as the trp lac fusions. In trp lac fusions which contain the end of the i gene, transcription starting at the trp promoter does not continue into the lactose structural genes but if the same fusion strain contains L1 transcription does continue into the lac genes.

It seems likely that termination of transcription normally occurs at the end of an operon. Messenger RNA corresponding to the

whole trp operon can be isolated from cells synthesising the trp biosynthetic enzymes. Whether RNA polymerase alone can terminate RNA chains at specific sites is not clear but in vitro experiments using ϕ80 d lac DNA, holoenzyme, crp protein and cAMP yield RNA molecules which are large and heterogeneous (no specific termination). Since in vitro conditions may not reflect the in vivo situation these results must be interpreted with caution.

a. Termination Factor

A protein (rho) has been isolated from E. coli (Roberts, 1969) which promotes the production in vitro of RNA molecules of discrete sizes (specific termination) from several template DNA's but as the in vivo concentration of rho is not known it is difficult to design in vitro experiments which mimic in vivo conditions.

A series of in vitro experiments using various concentrations of rho (de Crombrugghe, Adhya, Gottesman, Pastan, 1973) using lac and gal templates show that with low concentrations of rho (1 μgm/ml) specific termination at the end of the operons occurs. When the concentration of rho is raised (8 μgm/ml) termination at specific sites within the operons occurs. Whether this premature termination has any in vivo significance or is just an artefact produced by the high rho concentration is not clear. The in vivo function of rho can only be elucidated with the isolation of rho mutants.

b. Rho Action

Rho promoted termination leads to the release of RNA chains from the polymerase DNA complex but whether the polymerase is released is not clear.

Conflicting reports on whether rho binds to DNA, Hinkley quoted in Losick's Review (1972) says it does not but Oda and Takanami (1972) have published electronmicrographs of a ring of six rho subunits bound to phage fd DNA at a limited number of sites, make it difficult to choose between models for rho action. One model proposes that rho binds to specific sites on DNA thereby preventing transcription proceeding, another suggests that rho binds to polymerase conferring on it the ability to recognise rho termination signals. Rho does bind to polymerase (Hinkley, Losick's review, 1972) but which of the two models is correct will only be clarified with the isolation of rho mutants.

PART 2.

MATERIALS AND METHODS

a. General materials

Oxoid nutrient broth 2 Oxoid No. 2 powder 25g; distilled water 1 l.

L-broth (Lennox 1955) Difco tryptone 10g; yeast extract 5g; NaCl 10g; distilled water 1 l. pH 7-2

Minimal salts X4 (Meynell) NH_4Cl 20g; NH_4NO_3 4g; Na_2SO_4 8g; K_2HPO_4 12g; KH_2PO_4 4g; $MgSO_4\ 7H_2O$ 0.4g; distilled water 1 l.

M63 x 10 (Pardee, Jacob, Monod, 1959) K_2HPO_4 70g; KH_2PO_4 30g; $(NH_4)_2SO_4$ 20g; $FeSO_4$ 5 mg; distilled water 1 l.

After autoclaving add $MgSO_4\ 7H_2O$ 2g.

VB x 20 (Vogel, Bonner, 1956) $MgSO_4\ 7H_2O$ 4g; citric acid 40g; K_2HPO_4 200g; $Na\ NH_4HPO_4$ 70g; distilled water 1 l.

Buffer KH_2PO_4 3g; Na_2HPO_4 7g; NaCl 4g; $MgSO_4\ 7H_2O$ 0.2g; distilled water 1 l.

Oxoid nutrient agar oxoid NB2 plus Davis New Zealand agar 12.5 g/l.

Minimal agar minimal salts x 4 diluted 1:4 into molten solution Davis New Zealand agar (20g/l).

Amino acids Made as solution at 2 mg/ml and used at 20µg/ml.

Sugars Made as 20% solution and used at 0.2%.

Vitamin B_1 Made up at 1 mg/ml, used at 10µg/ml.

Streptomycin (Glaxo) Made up at 10mg/ml, used at 100µg/ml.

Spectinomycin (Upjohn) Made up at 10mg/ml, used at 100µg/ml.

Rifampicin (Lepetit S.p.A. Milan) Used at 200µg/ml for selection of spontaneous rif^r mutants, at all other times used at 100µg/ml. Stock solution for plates made at 100mg/ml in dimethylformamide (DMF) and stored at -20°C. Solution for experiments in liquid media made by dissolving powder in a few drops of DMF and then diluted to required concentration with vortexing in M63.

c-AMP (Sigma) adenosine 3':5' cyclomonophosphoric acid. Made up at 10^{-1}M in M63, filter sterilised, used at 10^{-3}M.

b. Indicator Media

EMB (Eosin, methylene blue) EMB peptone base (Difco casamino acids 42.4g; Difco yeast extract 5.2g; NaCl 27g; K_2HPO_4 27g; distilled water 1 l) 75mls; Eosin (4% solution in H_2O) 4mls; methylene blue (0.65% solution in H_2O) 4mls; desired sugar (20%) 20mls; Davis New Zealand agar (20g/l) 300 mls.

MacConkey Difco MacConkey No. 2 base 40g; distilled water 1 l. After autoclaving add desired sugar 10g/l.

Xg (5 bromo-4-chloro-3-indolyl-β-D-galactoside Cyclo Chemical) Xg made up at 20mg/ml in dimethylformamide

and 0.8ml added to 400ml bottle supplemented minimal agar with glucose as carbon source. When inducer was needed IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 10^{-3}M.

mms agar (methyl methane sulphonate Kodak)

Used for testing for recA⁻ marker.

Oxoid NB2 agar plus mms 0.05%. mms added to cool agar just before pouring. Plates used same day or next day if stored in refrigerator.

c. Special media

For P1$_{vir}$ lysates and transductions:

LC Bottom agar Tryptone 10g; Yeast extract 5g; NaCl 5g; Difco agar 10g; distilled water 1 l.

Before pouring to each 500ml bottle add LC mix 6.5ml.

LC mix 0.5M $CaCl_2$ 20ml; 20% glucose 25 ml; 0.25% thymidine 20ml.

LC Top agar Same as LC Bottom except Difco agar 7g;

Before use to each 100ml add 0.5M $CaCl_2$ 1.0ml.

Tryptone 1% in distilled water.

Adsorption medium 0.03M $MgSO_4$; 0.015M $CaCl_2$ in distilled water.

Citrated Buffer Buffer with trisodium citrate added to 5%

Citrated L-broth L-broth with trisodium citrate added to 1%.

For selecting thymine requiring strains

Trimethoprim (Burrows Wellcome) Used at 200µg/ml

in oxoid NB2 supplemented with thymine 100µg/ml

METHODS

a. Selection of Ara^+ medium

WZ 22 WZ 25 were grown overnight in oxoid NB2, 0.1 ml of the cultures were plated onto minimal arabinose, methionine B_1 plates. The plates were incubated at 30° for 48 hours. There were approximately 100 revertants per plate which gives a reversion rate of about $1x10^{-6}$ per bacterium plated. After purification Ara^+ colonies from four clones of WZ 22 and one clone of WZ 25 were tested for temperature sensitivity by plating on oxoid NB2 agar at 30° and 42°.

b. Hfr Matings

Donor and recipient cultures were grown into log phase (10^8 cells/ml) in oxoid NB2. 0.2ml of each culture were mixed and incubated at the appropriate temperature for the appropriate time. Controls were set up by mixing 0.2ml of each culture with 0.2ml of oxoid NB2 which were incubated with the mating mixtures. Dilutions of the mating and control mixtures were made in buffer and 0.1ml. plated on selective media.

c. F^- matings

a. The same method was used as for Hfr crosses.

b. A late log phase culture of the recipient was streaked down the middle of the selective plate and allowed to dry in. A

loopful of a log phase culture of the donor was then streaked across the recipient. This method provides its own controls.

d. P1 transductions

The method used was that of Willetts, 1967.

The recipient was grown in L-broth to 40 Klett units (red filter 660μm) approximately 10^8 cells/ml. The cultures were spun down and resuspended in 1/10 volume of 1% tryptone. 0.5ml of this suspension was mixed with 0.5ml adsorption mixture and 0.5ml of the P1 lysate diluted to 5×10^7 pfu/ml in L-broth. This gives a m.o.i. of 0.05 and ensures that multiple infections do not occur. Controls were done omitting phage to check reversion rate of bacteria, omitting bacteria to check sterility of lysate and omitting both to check sterility of media. The mixtures were incubated for 20 minutes at 37^o, spun down and washed twice with 5% citrated buffer, resuspended in 0.1ml citrated buffer and plated on selective medium with citrate added to 1%. The plates were incubated at the appropriate temperature for 2 days. Later transductions were done using Vogel/Bonner minimal medium, this medium contains citrate and eliminates the need to add citrate.

When the marker selected was str A or spc it was necessary to allow time for the expression of the recessive gene ($strA^r$ or spc^r). This was done by adding the incubated transduction mixture to 50mls of 1% citrated L-broth and growing the culture for 4-5 hours. 25mls of this culture was then spun down, resuspended in 0.5mls of citrated buffer and plated onto two selective plates.

c. β-galactosidase assay

Reagents

For β-galactosidase assays

Isopropyl-β-D-thiogalactopyranoside (IPTG) Sigma

Made up at 10^{-1}M in distilled water, autoclaved at 10 p.s.i. for 10 minutes. Used at 10^{-3}M.

o-Nitrophenyl-β-D-galactopyranoside (ONPG) Sigma

Made up at M/75 in M63.

PM2 (Reducing buffer of Revel, Luria, Rotman, 1961)

Na_2HPO4 5.1g; NaH_2PO_4 16.8g; distilled water 950ml; add 10^{-1}M $MgSO_4$ 10mls; 10^{-1}M $MnSO_4$ 2mls; β-mercapto-ethanol 6.8mls; make up to 1 l.

Cetyl triethylammonium bromide (CeTAB) (Tyler, Magasanik, 1969). 1mg/ml in distilled water.

Sodium deoxycholate (DOC)

1% in distilled water.

Sodium carbonate

1M solution in 8M urea.

Measurement of specific activity

Cultures were grown overnight in minimal medium with IPTG (10^{-3}M), diluted back in the same medium and grown into log phase. The cultures were then appropriately diluted in minimal medium plus CETAB 0.2mls, PM2 0.8mls and DOC 0.01ml to give a final volume of 2.0mls. The diluted cultures were kept in ice. The optical density of the cultures at 600μm was measured in a Zeiss spectrophotomete at the time of sampling. The samples were

assayed by adding M/75 ONPG 0.6mls and incubating at 28°C for a measured time. When enough colour had developed the reaction was stopped by adding 1M Na_2CO_3 in 8M urea 1.3mls. The optical density of the sample was measured at 420μm and 550μm using a no cell blank. Specific activity is expressed as

$$\frac{\text{O.D } 420\mu\text{m} - (\text{OD}550\mu\text{m} \times 1.75)}{\text{OD}600\mu\text{m} \times \text{time of incubation in minutes}}$$

b. Kinetics of enzyme synthesis

Sampling was done as for method a. Cultures were grown in sidearm flasks and the Klett value (red filter) was recorded at the time of each sampling. The enzyme was assayed as for method a, and the results expressed as a graph of enzyme activity/ml/min against Klett value at time of sampling.

f. Arabinose Isomerase Assay

The method used is essentially that of Cribbs and Engelsberg (1963).

Reagents

Low phosphate medium (Gross, Engelsberg, 1959)

KH_2PO_4 0.3g; K_2HPO_4 0.7g; $MgSO_4$ 0.1g; $(NH_4)_2SO_4$ 1g; casein hydrolysate 10g; distilled water 1 l. After autoclaving 15 p.s.i. 15 minutes add B1 10mg/l; glycerol 1g/l. For induction L-arabinose (filter sterilised) was added to a concentration of 6mM.

Assay Mix glycyl glycine buffer pH 7.6 75μM/ml;

$MnCl_2$ 2.5µM/ml; L-arabinose 150µM/ml.

Hydrochloric acid 0.1N.

Cysteine hydrochloride 1.5% in distilled water, made up fresh for each experiment.

Sulphuric acid 70%.

Carbazole 0.12% in 95% ethanol.

Cultures were grown overnight in low phosphate medium (Gross, Engelsberg, 1959) supplemented with glycerol 0.1% and arabinose 6mM. The cultures were diluted in the same medium to an optical density of approximately 6 Klett units (red filter). The cultures were grown to about 15 Klett units after which 1.0ml samples were taken, at recorded Klett values, into tubes at 0°C. Toluene, 1 drop, was added to each sample which was then vortexed for 15 seconds and stored at 0°C. To two 0.1ml aliquots of each sample was added assay mix 0.1ml and the reaction was allowed to proceed at 30°C for a measured time between 15 minutes and sixty minutes. The reaction was stopped by adding 0.1N HCl 1.8mls. An aliquot of the first sample in each series was incubated for 0 minutes before the addition of HCl to provide a blank for zeroing the Klett. The L-ribulose produced by the reaction was assayed by the cysteine carbazole method (Dische, Borenfreund, 1951). To each sample was added 1.5% L-cysteine (0.2ml) and 70% H_2SO_4 (6.0ml) and the mixture was then vortexed to ensure complete mixing. Carbazole solution 0.2mls was then added and the mixture was again vortexed. Colour was allowed to develop for at least 30 minutes, the colour produced was stable for at least another 40 minutes. The colour produced was

measured using a Klett (green filter 540μm) after zeroing the Klett to the blank sample. The results are expressed in graph form as Klett units/hour against Klett value at time of sampling.

Cribbs and Engelsberg (1963) express their results as enzyme units/mg total protein. A control experiment showed the relationship between the Klett value of the culture and total protein, assayed by the method of Lowry, Roseborough, Farr, Randall, (1951) to be linear in the range used and therefore measurement of total protein was not necessary.

As can be seen from figure 6b in results section, the curves of arabinose isomerase production do not extrapolate to zero as one would expect. The most simple explanation for this is that the addition of the 0.1N HCl to the blank sample does not stop the reaction instantaneously. This would mean that the blank sample is not a true zero but includes some increment due to ribulose production and so all the experimental values would appear too low by a constant amount.

g. RNA synthesis (Pato, Von Meyenberg, 1970)

Reagents

Oxoid NB2 plus uridine 40μg/ml.

M63 plus uridine 40μg/ml; methionine 20μg/ml; glucose 0.2%; B_1 10μg/ml.

Lysis mixture 0.1M NaCl; 0.01M Tris pH 7.5; 0.02M EDTA; 0.5% sodium dodecyl sulphate (SDS).

Carrier RNA yeast RNA 50μg/ml.

Trichloroacetic acid (TCA) 3M and 0.01M.

Millipore filters 25mm 0.45μ pore size.

Scintillant BBOT (Ciba) 4g/l toluene.

Uridine label 5' tritiated (Amersham) used at 2.5μCi/ml.

The method used is that described by Pato and von Meyenberg (1970).

Cultures were grown overnight at 30°C in oxoid NB2 plus 40 μg/ml uridine or M63 plus 40μg/ml uridine. Cultures were diluted back into same medium and grown to 25 Klett units (red filter) at 30°C. A 2.0ml sample was taken into a small flask and the main culture transferred to 42°C. To the 2.0ml sample at 30°C was added simultaneously ^{3}H uridine to a final concentration of 2.5μCi/ml and rifampicin to 200μg/ml. The uridine was labelled at the 5 position of the pyrimidine ring to minimise the entry of label into DNA. After the main culture had been growing at 42° for about one generation, a 2.0 ml sample was taken and labelled at 42°C in the same way as the first sample.

Samples from the labelling culture were taken and assayed as described by Bremer and Yuan (1968). Samples (0.1ml) were taken every 30 seconds for the first 6 minutes after addition of label and then every minute until 15 minutes after addition of label. The samples were taken into 0.5mls of lysis mixture at 100°C and were held at 100°C for 3 minutes. The samples were then cooled to room temperature 1.0ml of a 50μg/ml solution of yeast RNA was added as carrier. The RNA was then precipitated with 0.5ml of 3% TCA. The RNA was then collected on Millipore filters which were washed with

4 x 5ml 0.01M TCA dried and counted using BBOT scintillant in a Packard counter.

h. Assay for rel status

The method used was a modification of that described by Fill(1969).

Reagents

M63 supplemented with glucose 0.2%; B_1 10µg/ml methionine 20µg/ml; arginine 20µg/ml; thymine 100µg/ml; uridine 40µg/ml.

Uridine and Leucine labels 5' ^3H uridine 2.5µCi/ml ^{14}C leucine 0.01 µCi/ml.

TCA 5%.

Ethanol 70%.

Scintillant BBOT (CIBA) in toluene 4g/l.

Millipore filters 25mm 0.45µ pore size.

Cultures were grown overnight in M63 supplemented with glucose 0.2%; methionine and arginine 20µg/ml; thymine 100µg/ml; and uridine 40µg/ml. Cultures were diluted fifty-fold in the same medium and grown for four hours at 30°C with shaking. Two 1.0ml samples from each culture were spun down, washed twice in the same medium without methionine, resuspended in 1.0ml of the same medium without methionine and incubated at 30°C for twenty minutes. ^{14}C leucine 0.01µCi/ml and ^3H uridine 2.5µCi/ml were then added and the cultures incubated at 30°C for a further thirty minutes. Ice cold 5% TCA, 5mls, was then added to each culture to precipitate proteins

and nucleic acids and the preparation allowed to stand for twenty minutes. The precipitated proteins and nucleic acids were collected on a millipore filter, washed with 4 x 5ml 5% TCA and 1 x 10ml 70% ethanol. The filters were then dried and counted with BBOT scintillant in a Packard counter.

The ratio of ^{14}C counts in protein to ^{3}H counts in RNA for a rel$^-$ control is expressed as 1:1. Under these conditions the ratio for a rel$^+$ strain is between 5 and 8:1.

i. Selection of thyA$^-$ and low thymine requiring strains

To 5mls of oxoid NB2 supplemented with thymine 100µg/ml and trimethoprim (200µg/ml), was added 5 x 10^6 bacteria. The culture showed little visible growth after 24 hours. After 48 hours growth the culture was streaked onto oxoid agar supplemented with thymine 100µg/ml and trimethoprim (200µg/ml). Single colonies from this plate were streaked onto minimal glucose plates with and without thymine 100µg/ml, thyA$^-$ clones grow only on the plate with thymine.

To select a low thymine requiring strain a thyA$^-$ strain is streaked onto a minimal plate with thymine 10µg/ml added and clones which grow on this plate but not a plate without thymine are low thymine requirers.

III Construction of strains

Markers are mutant alleles unless marked $^+$.

WZ11 metB, strA, B_1 (strA derivative of W1655 Lederberg and Lederberg 1953).

WZ22 metB, strA, B_1 cya855 Selected as Lac$^-$ on lactose tetrazolium after UV mutagenesis (T. Tsuji unpublished).

WZ25 metB, strA, B_1 crp868. Same selection as WZ22.

WZ30 metB, strA, B_1 cya855 alt-1. Spontaneous Ara$^+$ of WZ22 selected on minimal arabinose at 30°C (2.II.a).

WZ31 metB, strA, B_1 crp868 ts2. Spontaneous Ara$^+$ of WZ25 selected on minimal arabinose at 30°C. (2.II.a).

WZ33 thyA derivative of WZ30 by trimethoprim method (2.I.i).

WZ57 metB$^+$ cya855$^+$ alt-1 strA B_1. WZ30 mated with D8007 (transfers cya$^+$ early, see fig. 1, section 2.V) selecting Met$^+$ recombinants. Met$^+$ recombinants checked for alt-1 by streaking on oxoid agar at 30°C, 42°C and for cya$^+$ by their irreversibility by 10^{-3}M cAMP.

WZ61 metB, argA cya855 alt-1 rel$^+$ (strains derived from W1655 are rel$^-$). WZ33 P1 transduced with lysate from NF58 (thyA$^+$ argA rel$^+$) selecting Thy$^+$ transductants. Transductants were checked for cotransduction of ArgA, 27/100 were also argA. Ten thyA$^+$ argA were checked for rel status and 4 were rel$^+$. In a parallel cross using a lysate from an isogenic rel$^-$ strain 18/100 thyA$^+$ were argA and 10/10 of these were rel$^-$.

WZ84 metB cya855 crp868 strA spc alt-1 B_1 WZ30 P1 transduced with a lysate from WZ83 (argG metB his ylacproXIII strA spc amp crp868 cya855) selecting Spc^r. 23 transductants were purified and then streaked onto minimal, arg, lac, xyl, gly at 30°C and 42°C. 3/23 grew on min ara but not lac, xyl and gly at 30°C, none of the 23 grew on any of the carbon

sources at 42°C. One of the 3 which was Ara$^+$ Lac$^-$ at 30°C was shown to contain crp868 in two ways. Firstly it could give Lac$^+$ in the presence of cAMP only when infected with KLF41 which covers the crp region (Fig. 1 section 2.IV), secondly the crp marker could be rescued with the phage P1 into WZ11 yielding Spc^r Ara$^-$ transductants.

WZ86 cya$^+$ crp868 alt-1 strA spc WZ57 P1 transduced with lysate from WZ83 selecting Spc^r. 4/32 Spc^r were Ara$^+$ Lac$^-$ Xyl$^-$ Gly$^-$ at 30°C and did not grow on any of these sugars at 42°C. The Lac$^+$ phenotype could be restored by infection with KLF41.

WZ93 metB cya855 crp868 alt-1 spc strA glp$^+$. Spontaneous Gly$^+$ selected from WZ84 as a red sector on MacConkey glycerol agar. The strain remained Ara$^+$, Lac$^-$, Xyl$^-$ and alt-1. The glp$^+$ marker maps near metB, 12/16 Met$^+$ transductants using P1 lysate from CA8000 (wild type strain) were also Glp$^-$. These figures are in good agreement with Berman (1971) for the cotransduction of glyK with metB. This glp$^+$ mutation is possibly a glyK promoter mutation making it independent of the crp-cAMP system.

WZ94 cya$^+$ crp868 alt-1 glp$^+$ strA. Spontaneous Glp$^+$ derivative of WZ86 by same method as WZ93. The strain remained Ara$^+$ Lac$^-$ Xyl$^-$ alt-1. The strain could not be checked for cotransduction of the glp$^+$ marker with metB as the strain is already metB$^+$ but there is no reason to suppose it is not similar to the WZ93 mutation.

WZ81 (*argG*⁻ *metB*⁻ *cya*⁻ *crp*⁻ *spc*ʳ *str*ʳ *amp*ʳ *recA56*⁻)

WZ37 (JC411, *his*⁻ *leu*⁻ *metB*⁻ *argG*⁻ *str*ʳ *Su*⁺)

→ P1 transduction with lysate from WZ38 (*metB*⁻ *argG*⁻ *str*ˢ *spc*ʳ *Su*⁺) selecting Spc^r screened for *str*ˢ.

Done by

AJ102 (*his*⁻ *leu*⁻ *argG*⁻ *metB*⁻ *str*ˢ *spc*ʳ *lacy*⁻)

A.B. Silverstone

→ Mated with D8016 Hfr Cavalli (*metB*⁻ *cya*⁻ *amp*ʳ *str*ˢ selecting amp^r, screened for Ara⁺ Leu⁻.

WZ70 (*metB*⁻ *argG*⁻ *his*⁻ *leu*⁺ *spc*ʳ *str*ˢ *amp*ʳ *cya*⁻ *lacy*⁻)

→ Mated with CA8000 (wild type) selecting Lac⁺ on minimal lactose + c-AMP.

WZ72 (*argG*⁻ *metB* *cya*⁻ *his*⁻ *spc*ʳ *str*ˢ *amp*ʳ *lacy*⁺)

- P1 transduction with lysate from WZ25 (*crp*⁻ *str*ʳ) selecting Str^r, screened for *crp*⁻, only get Arg⁺ Ara⁺ recombinants with KLP41 in presence of cAMP.

WZ75 (*argG*⁻ *metB*⁻ *his*⁻ *cya*⁻ *crp*⁻ *spc*ʳ *str*ʳ *amp*ʳ)

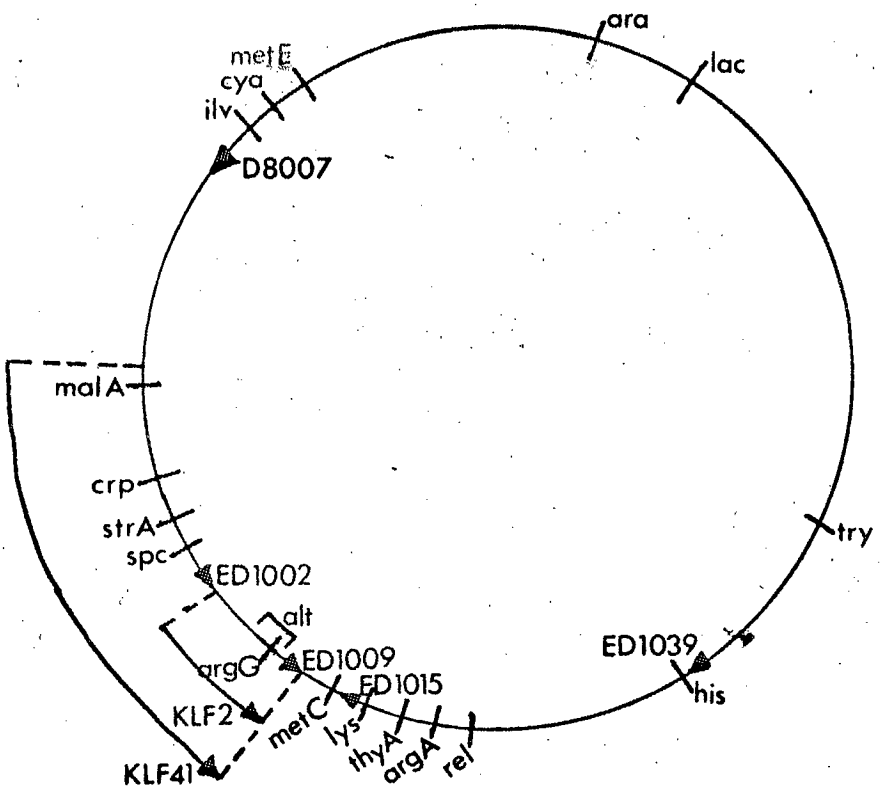
- Mated with JG47 (HfrKL16 *thr*⁻ *ilv*⁻ *recA56*⁻ *spc*ʳ *str*ʳ) selecting His⁺ 3/8 had also become MMS sensitive (*rec*⁻).

WZ81 (*argG*⁻ *metB*⁻ *cya*⁻ *crp*⁻ *spc*ʳ *str*ʳ *amp*ʳ *recA56*)

IV Strain genotypes and source

Strain	Genotype. All markers are mutant alleles except where shown $^{+}$.	Source
WZ11	metB, strA, rel	W1655. Lederberg, Lederberg, 1953.
WZ22	metB, strA, rel, cya855	UV on WZ11. T. Tsuji
WZ25	metB, strA, rel, crp868	UV on WZ11. T. Tsuji
WZ30	metB, strA, rel, cya855, alt-1	Ara^{+} revertant-WZ22
WZ31	metB, strA, rel, crp868, ts-2	Ara^{+} revertant-WZ25
WZ33	metB strA rel cya855 alt-1 thyA	thyA WZ30
WZ35	metB strA rel cya855 alt-1 thyA	Low thymine requiring derivative WZ33
WZ57	strA rel alt-1	WZ30 x D8007 select Met^{+}
WZ61	metB, strA, cya855, argA, rel^{+}	WZ33 PIMF58 select thy^{+}
WZ81	metB, argG, cya855, crp 868, strA, spc, amp, recA56	From JC41
Wz83	metB argG, cya855, crp868, strA, spc, amp, his, ViacproXIII	From JC411
WZ84	metB, strA, cya855, crp868, alt-1, spc, rel	WZ30 P1 WZ83 select Spc^{r}
WZ86	strA, crp868, alt-1, spc, rel	WZ57 P1 WZ83 select Spcr
WZ93	metB, strA, cya855, crp868, alt-1, spc, rel, glp^{+}	Glp^{+} revertant WZ84
WZ94	strA, crp868, alt-1, spc, rel, glp^{+}	Glp^{+} revertant WZ86

Strain	Genotype. All markers mutant alleles except where shown +	Source
KLF2	leu⁻ argG⁻ his⁻ metB⁻ lac⁻ strA recA56⁻/KLF2	Low 1968
KLF41	leu⁻ argG⁻ his⁻ metB⁻ lac⁻ mal⁻ xyl⁻ strA $recA_1$⁻/KLF41	Low 1968
CA8000	HfrH lac⁺ strA	Beckwith
ED1002	Hfr Vlac proXIII integrated F+s lac spc str^s	Masters Broda 1971
ED1009	Hfr Vlac proXIII integrated F+s lac spc str^s	Masters Broda 1971
ED1015	Hfr Vlac proXIII integrated F+s lac spc str^s	Masters Broda 1971
ED1019	Hfr Vlac proXIII integrated F+s lac spc str^s	Masters Broda 1971
D8007	Hfr G10 his str^s	Matney Erwin Goldschmidt 1963
NF58	F⁻ metB argA rel⁺ W1655 derivative	FIIL 1969
NF59	F⁻ metB argA rel W1655 derivative	FIIL 1969
JG47	Hfr KL16 thr his recA56 spc strA	Gross


ara
lac
metE
cya
ilv
D8007
malA
crp
strA
spc
ED1002
alt
argG
ED1009
ED1015
KLF2
metC
lys
thyA
argA
rel
KLF41
try
ED1039
his

V. Linkage map of E. coli showing relevant markers and direction and origin of Hfrs and P^1's used (Fig. 1)

Genetic loci follow standard abbreviations (Taylor, Trotter, 1972). cya is the genetic locus for adenyl cyclase (Yokota, Gots, 1970); crp is the locus for the gene producing a protein necessary for the transcription of catabolite sensitive operons (Schwartz, Beckwith, 1970; Perlman et al, 1970); alt is the locus for the temperature sensitive factor discussed in text.

The P^1 KLP2 does not transfer strA (Low, 1968). The KLP2 donor B11012 (leu$^-$ his$^-$ argG$^-$ metB$^-$ strA^r spc^r/P^1 argG$^+$ argR$^+$) was checked for transfer of strA by mating it with WZ38 (metB$^-$ argG$^-$ str^s spc^r Su$^+$) and selecting Arg$^+$ recombinants on minimal glucose metB$_1$ plates, the donor was selected against with the leu$^-$ his$^-$ markers. If KLP2 transferred strA^r then recombinant Arg$^+$ clones should throw off str^r clones by homogenotisation between the chromosomal str^s and the episomal str^r markers. Four independent Arg$^+$ clones were tested by streaking them onto oxoid plates with and without streptomycin, none of them gave any StrA^r colonies. The position of the crp gene was confirmed to map near strA in the construction of strain WZ80 (F$^-$ his$^-$ argG$^-$ metB$^-$ Vlac proXIII strA^r spc^r amp^r cyc855$^-$ crp868$^-$) and also close to spc in construction of WZ84. The crp$^-$ marker was P1 cotransducible with strA. No accurate figure could be put on the cotransduction frequency due to the need to allow for phenotypic lag in the expression of streptomycin resistance, one cannot therefore be sure that all the clones selected originated from independent transductional events. A technique was developed for the easy identification of crp$^-$ transducts. Streptomycin resistant transductants were selected on arabinose tetra-

zolium c-AMP streptomycin plates. Clones which have also become crp$^-$ will appear as dark red as they will not be made Ara$^+$ by the cAMP. Of four colonies picked as dark red all four were crp$^-$ as shown in that the Ara$^+$ phenotype can be restored if the strain is infected with KLP41 in the presence of c-AMP. Since when selecting only for streptomycin resistance only about 10% of these become also crp$^-$ this method is obviously very efficient in obtaining crp$^-$ transductants.

The crp868$^-$ strain WZ25 has been shown to have at least a six-fold reduction in c-AMP binding activity as compared with WZ11 (Ellen, Henderson, personal communication).

The cya855$^-$ strain WZ32 has at most a very low level of c-AMP (5%) and possibly none at all (Silverstone, Goman, Scaife, 1972).

PART 3 EXPERIMENTAL STUDIES

I. Experimental Rationale

Since *crp*$^-$ and *cya*$^-$ mutations affect the expression of genes at the level of transcription they should be a good starting point for the selection of mutations affecting transcription in other ways.

Ara$^+$ revertants selected from a *cya*$^-$ or a *crp*$^-$ strain could arise in a number of ways. They could arise by back mutation to *cya*$^+$ or *crp*$^+$, or by mutations of the *UV5* type (Section 1,II,g) in the *ara* promoter circumventing the need for the crp cAMP complex. The first class can be eliminated by showing that the reversion mutation does not map in the genes *crp* or *cya*, the second by showing that the reversion is pleiotropic for other sugars as a mutation in the ara promoter will not affect expression of other operons.

A third class which will be pleiotropic for the utilisation of a number of sugars can be divided into two major subclasses. A subclass with a change in a subunit of the RNA polymerase so that it can recognise promoters more efficiently without the crp-cAMP complex. An alteration in a protein so that it can stimulate polymerase binding either in a way analogous to sigma or the crp cAMP system.

It is also possible that this selection will provide mutations which affect the translational process so that the low level of mRNA made in *crp*$^-$ or *cya*$^-$ strains (2% wild type) will be more efficiently translated giving a high enough level of the arabinose enzymes to permit growth.

Ara$^+$ revertants of *crp*$^-$ or *cya*$^-$ mutants could therefore be a rich source of secondary mutations of great relevance to our

understanding the mechanism of gene expression. I also reasoned that investigation of the secondary mutations would be much easier if they conferred an additional property on the cell as a whole. I therefore undertook to seek Ara^+ revertants whose growth on rich medium was inhibited at 42°C.

II. Isolation of Ara^+ revertants from cya^- and crp^- strains

Revertants of cya^- and crp^- strains were isolated on appropriately supplemented minimal medium containing arabinose as sole carbon source (Section 2.II.a). Ara^+ revertants of WZ22 (cya^-) were tested for temperature sensitivity by plating for single colonies on oxoid agar at 30°C and 42°C. Four independent clones gave respectively 2/11, 1/32, 4/32 and 3/32 temperature-sensitive Ara^+ revertants, showing that the Ara^+ phenotype can be commonly restored by mutation(s) concomitantly rendering the strain temperature-sensitive.

Ara^+ revertants of one clone of WZ25 (crp^-) were tested and 3/8 were temperature sensitive. All the temperature sensitive revertants had regained the ability to express other catabolite sensitive genes becoming Lac^+ Xyl^+ Mal^+ Glp^+. Some of the temperature resistant revertants were positive only for arabinose utilisation and are probably mutants in the ara promoter circumventing the need for the crp c-AMP system.

III Genetic characterisation of alt-1 and ts2

For reasons which will become clear later in this thesis the ts^- mutation in WZ30 (cya^- ts^-) has been called alt-1 (alternative pathway for the expression of catabolite sensitive genes). The temperature

sensitive mutation in WZ31 (*crp*$^-$ *ts*$^-$) has been called *ts2* as it has not definitely been established to be a mutation of the *alt* class.

a. *Hfr mapping*

In order to prove that the *alt-1* and *ts2* mutations chosen for study were not in the *crp* or *cya* genes, it was necessary to show that they map at different points on the chromosome. Approximate mapping of the *alt-1* and *ts2* loci was done using four Hfr strains (Masters, Broda, 1971) ED1002, ED1009, ED1015 and ED1039 whose origins and direction of transfer are shown in figure 1 (section 2.IV). This preliminary mapping placed both *alt-1* and *ts2* between the origins of ED1009 and ED1002, (Table 1) between 61 and 65 minutes of the chromosome.

As selecting streptomycin resistant recombinants may influence the recombinants obtained by forcing recombination events to occur in the region of the Hfrs transferred very early (Hfrs are streptomycin sensitive) it was decided to check WZ30 using a method not dependent on a streptomycin counterselection. From crosses of WZ30 against Hfrs, ED1009 and ED1002 dilutions were plated onto minimal glucose B_1 plates, on which neither the *pro*$^-$ donors nor the *metB*$^-$ recipient will grow. The Met$^+$ recombinants were selected at 30°C. In the cross WZ30, ED1009 the majority (69/105) of the MetB$^+$ recombinants were also *Ts*$^+$. By contrast none (0/105) of the MetB$^+$ recombinants in the cross WZ30, ED1002 were Ts$^+$.

To ensure the validity of these results and to prove that the *alt-1* locus is not in the *crp* gene the origins and ability to transfer *crp*$^+$ of the Hfrs ED1002 and ED1009 was checked. Table 2

shows that strain ED1009 transfers argG early followed by metB whereas ED1002 transfers argG at a low frequency as a terminal marker. When Met$^+$ recombinants of a cross WZ25 (crp$^-$ metB$^-$) ED1002 are obtained a large proportion of them (90/109) also become Lac$^+$ Ara$^+$ and therefore must be crp$^+$. Since we know that the cya marker maps close to metB (Yokota, Gots, 1970) and that ED1002 transfers crp$^+$ early but not alt-1 then alt-1 cannot be in either the cya or crp genes. The alt-1 (and ts2) mutations map in the argG region of the chromosome between the origins ED1002 and ED1009.

b. F prime mapping

To check the position of the alt-1 marker in WZ30 and ts2 marker in WZ31 they were mated against a KLF2 donor. This is an F factor carrying the argG region of the chromosome (Fig. 1, Section 2. V). The strain carrying the KLF2 episome is recA$^-$ thus it will only transfer the episome since Hfrs are very rarely generated in recA$^-$ strains. Consequently if the KLF2 donor gives Ts$^+$ recombinants with alt-1 and ts2 these mutations must be in the small chromosomal segment on the episome. The episome KLF2 transfers alt-1 ts2 and argG to suitable recipients in comparible numbers (table 3) showing that the alt-1 and ts2 mutations both map in the small region of the chromosome carried by KLF2.

TABLE 1

Transfer of ts^+ by Hfr's ED1002, ED1009, ED1015 and ED1039

	RECIPIENT	
	WZ30 (cya^- alt1)	WZ31 (crp^- ts2)
DONOR		
ED1002	-	-
ED1009	+	+
ED1015	-	-
ED1039	-	-

+ = ts^+ recombinants which were at least a hundred-fold higher than

- = no ts^+ recombinants

Mating mixtures were incubated for 30' at 30°C, diluted, spread on oxoid agar plus streptomycin (to select against donors) and incubated at 42°C.

TABLE 2

Transfer of $metB^+$ $argG^+$ of Hfr's ED1002 and ED1009

	RECOMBINANTS OBTAINED PER ML OF THE MATING MIXTURE.	
DONOR	Arg^+	Met^+
ED1009	1.5×10^6	1×10^5
ED1002	1.5×10^4	2×10^5

The two donors were mated against WZ37 (*his*$^-$ *leu*$^-$ *metB*$^-$ *argG*$^-$ *strA*^{r} *su*$^+$). Mating mixtures were incubated for 30' at 37°C, dilutions were plated onto minimal glucose his leu metB, to select for Arg^+ and minimal glucose his leu arg B_1 for Met^+. Donors were selected against by the absence of proline from the medium.

TABLE 3

Transfer of TS^+ Arg^+ by KLF2 donor

Recipient strain	Treatment	Phenotype selected	0.1ml of culture plated after dilution. 10^0	10^{-1}	10^{-2}
WZ 30	mated	TS^+	>1000	97	2
WZ 30	unmated	TS^+	27	2	1
WZ 31	mated	TS^+	>1000	99	7
WZ 31	unmated	TS^+	28	4	2
WZ 37	mated	Arg^+	uncountable	500	1
WZ 37	unmated	Arg^+	0	0	0

KLF2 donor (E11012) his⁻ leu⁻ metB⁻ argG⁻ recA⁻56 $strA^r$/F'$argG^+$ $argR^+$

Recipients: WZ 30 cya^- alt1 $metB^-$ $strA^r$

WZ 31 crp^- tS2 $metB^-$ $strA^r$

WZ 37 his⁻ leu⁻ $metB^-$ argG⁻ $strA^r$ su^+

Mating mixtures were incubated for 2 hours at 30°C.

TS^+ recombinants were selected on minimal glu, met, B_1 plates, donor counterselected against with leu^- his^- markers Arg^+ recombinants selected on minimal glucose, his, met, leu, B_1 MMS plates (MMS, methyl methane sulphonate selects against donor as $recA^-$ strains do not grow on this compound).

WZ 30 and WZ 31 control numbers are obtained by plating dilutions of unmated cultures of these strains.

c. Transfer of alt1 to KLF2 by homogenotisation

In order to manipulate the alt-1 marker in WZ30 I tried to isolate a KLF2 episome carrying alt-1. This was done by introducing KLF2 into WZ30 (section 3,III,b). Sixteen independent temperature resistant clones were thus obtained. They were purified and then streaked to single colonies on oxoid agar at 30°C. Each of these should have the genotype alt-1/KLF2 alt$^+$ but should occasionnally give rise to alt-1/KLF2 alt-1 homogenotes. On mating, a homogenote should be able to convert a cya$^-$ crp$^-$ recA$^-$ strain to Ara$^+$ at 30°C solely by transfer of its recombinant KLF2. Note that, except under the special circumstances discussed below, a recA$^-$ strain cannot generate recombinants for extra-episomal chromosomal genes mobilised by KLF2. The yield of derivatives inheriting the autonomous F-prime factor, on the other hand, is virtually unaffected by the recA$^-$ mutation. Thus the donor culture should give Ara$^+$ recombinants at a low frequency reflecting the number of alt-1/KLF2 alt-1 homogenotes in the population. A colony from each of the sixteen TS$^+$ clones was tested for transfer of Arg$^+$ and Arg$^+$Ara$^+$ to WZ81 (argG$^-$ metB$^-$ cya$^-$ crp$^-$ spc^r strA^r amp^r recA56$^-$). Twelve of the sixteen TS$^+$ clones gave Arg$^+$ recombinants, the other four, which could not were probably TS$^+$ revertants of WZ30. Six of the Arg$^+$ donors gave Arg$^+$ Ara$^+$ recombinants. The Arg$^+$ Ara$^+$ recombinants occured at a much lower frequency (less than 0.1%) than the Arg$^+$ recombinants as expected if each colony contained only a small proportion of alt-1/KLF2 alt-1 homogenotes. Of eight Arg$^+$ recombinants tested all were met$^-$ recA$^-$. By contrast of eight Arg$^+$ Ara$^+$ recombinants seven were also rec$^+$, (like the donor and recipient they were all still met$^-$) implying that in order to become Ara$^+$ the

recombinants must receive *rec*$^+$ from the donor. Apparently, Ara$^+$ recombinants can only be obtained if part of the donor chromosome is inherited by WZ81, with a consequent necessity for the recombinant cells to become at least transiently *recA*$^+$. This suggests that for *alt-1* to be expressed, it must be associated with a second mutation outside the chromosomat segment of KLF2. It should be remembered at this point that WZ81 is derived from WZ37 (JC411) whereas the *alt-1* mutation was isolated in a W1655 strain. It may be possible that W1655 harbours a cryptic gene, necessary for *alt-1* expression that is different in WZ37 strains. This anomaly does not basically affect the results shown later of the effects of the *alt-1* mutation on RNA synthesis and the expression of catabolite sensitive genes. The cryptic gene need not necessarily have been mutated to permit expression of the Alt-1 phenotype. The frequency at which TS$^-$ revertants arise (1×10^{-6}) argues against a double mutation having occured. I intend to try and establish and map the second locus.

The second locus could have been mutated during the isolation of WZ22 which was isolated after UV mutagenesis of WZ11. If this is true, it should be possible to cross it out with a P1 lysate of WZ11 (see discussion section 4).

IV The alt 1 mutation affects gross RNA synthesis

a. The effect of alt 1 on growth

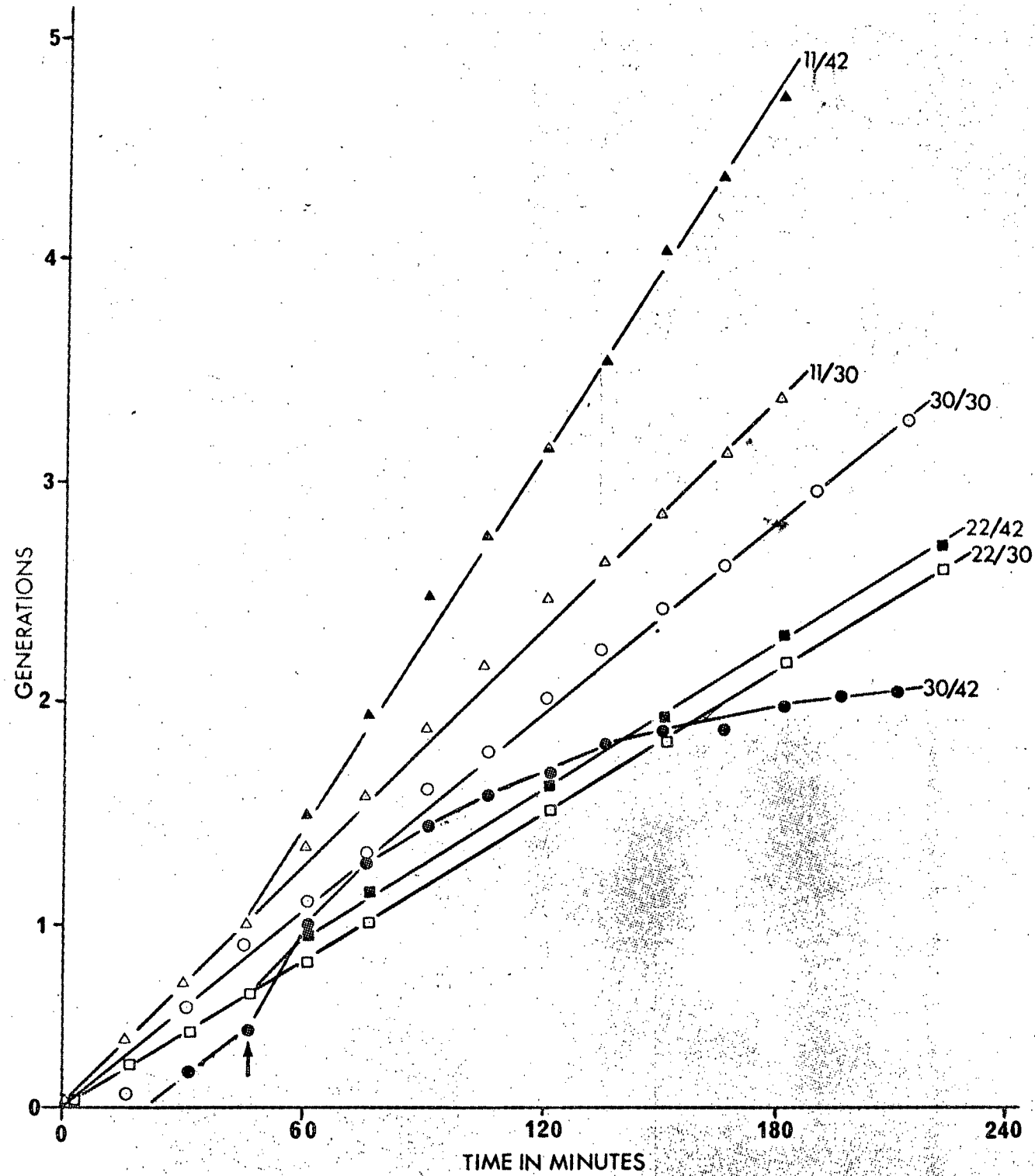
The strains WZ30 (*cya*$^-$ *alt-1*) and WZ31 (*crp*$^-$ *TS2*)cannot form single colonies on rich or minimal agar at 42°C. The temperature sensitivity of strain WZ30 during growth in nutrient broth (Oxoid) is shown in figure 2. Its growth begins to slow down after about one generation while WZ11 (*cya*$^+$ *alt*$^+$) and WZ22 (*cya*$^-$ *alt*$^+$) are not

inhibited after two or three generations. The temperature sensitive revertant (tS2) of WZ25 (crp⁻) also shuts off in the same time when grown in Oxoid broth (not shown). When strains WZ30 and WZ31 are grown in liquid minimal medium growth does not show any inhibition after a shift to 42°C for three or four generations. Nevertheless, they do not form colonies when grown on minimal agar. The temperature sensitive lesion seems to involve metabolic processes which are used for growth in rich medium (catabolic) but at least one essential function operating in both rich and minimal media must be affected.

b. The effect of alt 1 on DNA and RNA synthesis

As it was hoped that our selection procedure would provide mutations which affected RNA synthesis it was necessary to see if alt-1 was in fact an RNA synthesis mutation. Accumulation of radioisotope into DNA and RNA indicates that RNA synthesis is primarily affected by the alt-1 mutation (Figure 3). Following a shift to 42°C accumulation of ^{3}H into RNA in WZ35 (a low thymine requiring derivative of WZ30 cya⁻ alt-1) is severely reduced in less than a quarter of a generation while DNA synthesis continues for nearly a whole generation. From this experiment it is clear that RNA synthesis is affected before DNA synthesis.

Is the alt-1 product directly involved in RNA synthesis? Studies reported in subsequent sections of this thesis lead us to believe it is.


11/42
11/30
30/30
22/42
22/30
30/42
GENERATIONS
TIME IN MINUTES
0
1
2
3
4
5
0
60
120
180
240

Legend to Figure 2

Growth of strains in Oxoid broth

The strains were grown in oxoid broth in side-arm flasks at 30°C, at the point indicated by the arrow half of each culture was transferred to a pre-warmed flask and incubated at 42°C. Growth of the cultures was followed using a Klett colorimeter with a red filter (660mμ). During the course of the experiment cultures were diluted back into pre-warmed medium to keep them growing exponentially.

▲ WZ11 at 42°C.

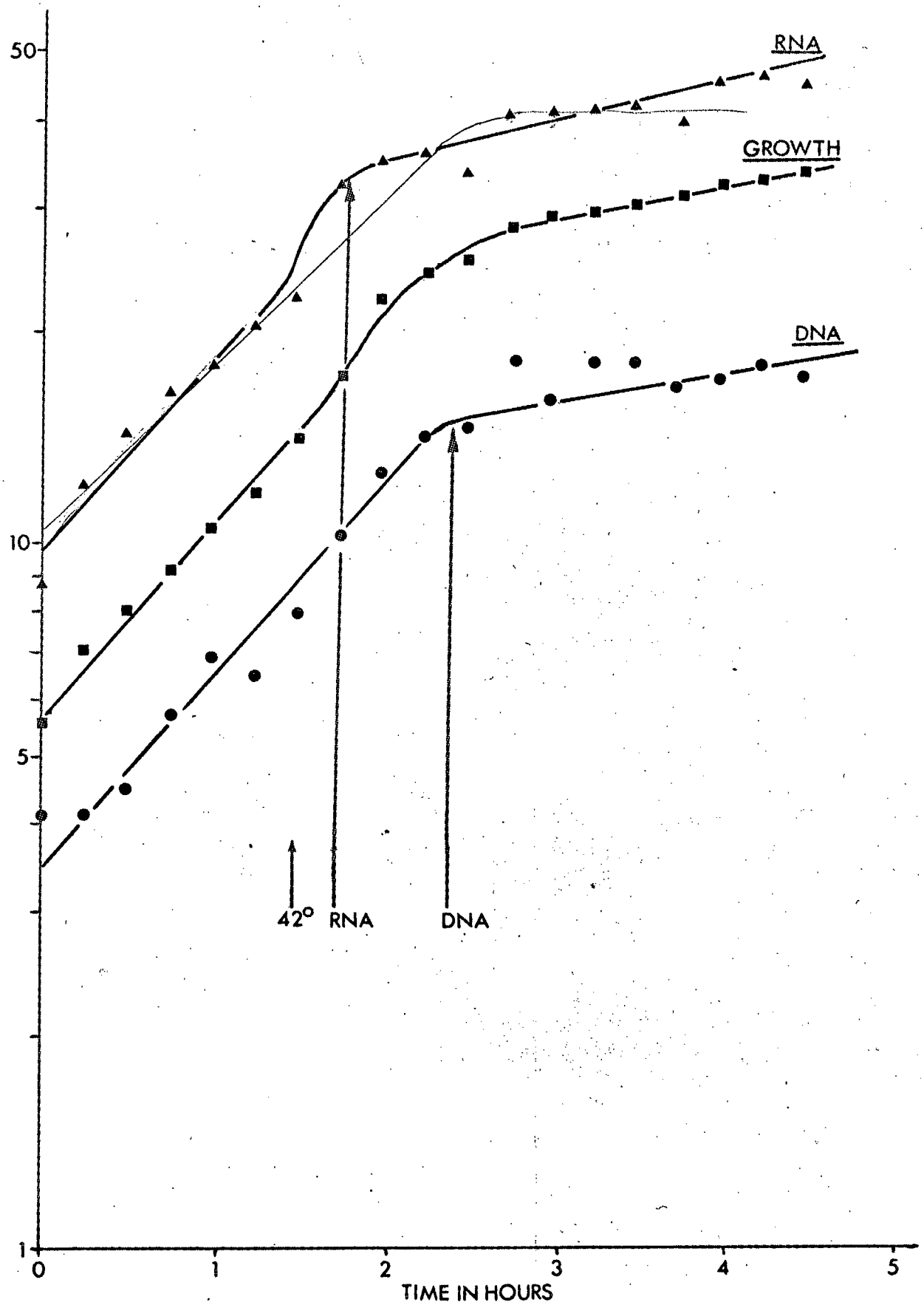
△ WZ11 at 30°

● WZ30 at 42°

○ WZ30 at 30°

■ WZ22 at 42°

□ WZ22 at 30°


RNA
GROWTH
DNA
50
10
5
1
0
1
2
3
4
5
42°
RNA
DNA
TIME IN HOURS

Legend to Figure 3

Continuous labelling of RNA and DNA in WZ35

WZ35 is a low thymine requiring (10μgm/ml) derivative of WZ30, it was obtained as described in methods. A low thymine requiring strain is necessary to ensure adequate entry of ^{3}H thymine into DNA. Strains which are thy^{+} do not take up thymine and therefore DNA is not labelled.

Scales on the ordinate are:

Growth in Klett units (red filter)
RNA in ^{3}H counts per 20′ x 10^{-4}
DNA in ^{3}H counts per 20′ x 10^{-3}

WZ35 was grown overnight at 30°C in Oxoid N132 plus 10μg/ml thymine. The culture was diluted into three flasks one containing Oxoid NB2, 10μg/ml thymine, one containing Oxoid NB2, 10μg/ml thymine ^{3}H uracil (.25 μCi/ml) and one containing Oxoid NB2, thymine 10μg/ml, ^{3}H thymine (1.0μCi/ml). The cultures were grown for several generations at 30°C to obtain a linear rate of entry of labelled precursors and then diluted in the same media to about 5 Klett units. The optical density of the culture without label was followed on a Klett colorimeter.

At intervals 0.1 ml samples of the labelled cultures were applied to 3MM, 2.4 cm Whatman filter discs which were immediately dropped into a large volume of ice-cold 5% TCA. After two hours in the TCA the filter discs were washed twice with acetone, dried, and counted in BBOT scintillant in a Packard Tri-carb liquid scintillation counter. At the point indicated by the arrow all three cultures were switched to 42°C.

c. The alt 1 product preferentially affects synthesis of unstable RNA species.

The technique of Pato and Von Meyenberg (1970) enables one to distinguish between stable and unstable RNA synthesis in vivo. Following the simultaneous administration of ^{3}H-uridine and rifampicin to a growing (rif-s) bacterial culture only those RNA polymerase molecules that have started transcribing are able to incorporate label into RNA. This is because the rifampicin inhibits further initiation of transcription. Thus RNA synthesis ceases after rounds of transcription in progress have been completed. The peak, which is reached in 2-4 minutes, contains both a stable and an unstable RNA fraction. The unstable fraction is broken down until a plateau is reached which corresponds to the stable fraction, the plateau is reached in about 10 minutes.

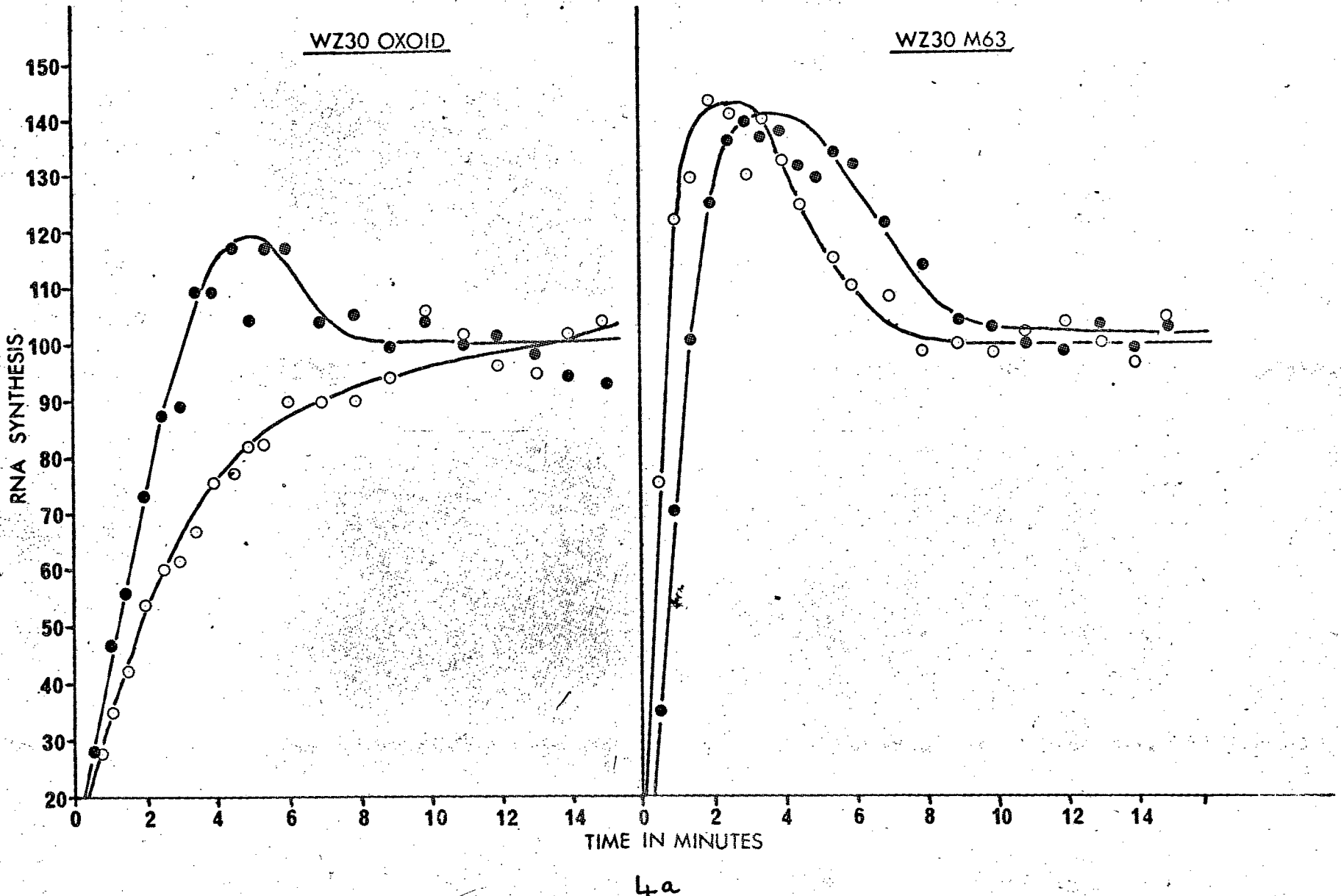
The synthesis of stable and unstable RNA species in an alt-1 strain was measured by this technique. I compared their synthesis before and after switching the growing culture to 42°C. The effect of the temperature shift on WZ30 (cya^{-} alt-1) growing in booth is shown in Figure 4a. It can be seen that following the temperature shift we no longer see the peak of unstable RNA. I attribute the loss of the unstable peak to the alt-1 mutation since the alt^{+} parent of WZ30 (WZ22) continues to synthesise normal amounts of unstable RNA at 42°C in broth, Figure 4b.

Thus a major species of unstable RNA either depends for its synthesis on an intact alt-1 product, or is degraded much faster when alt-1 is inactive.

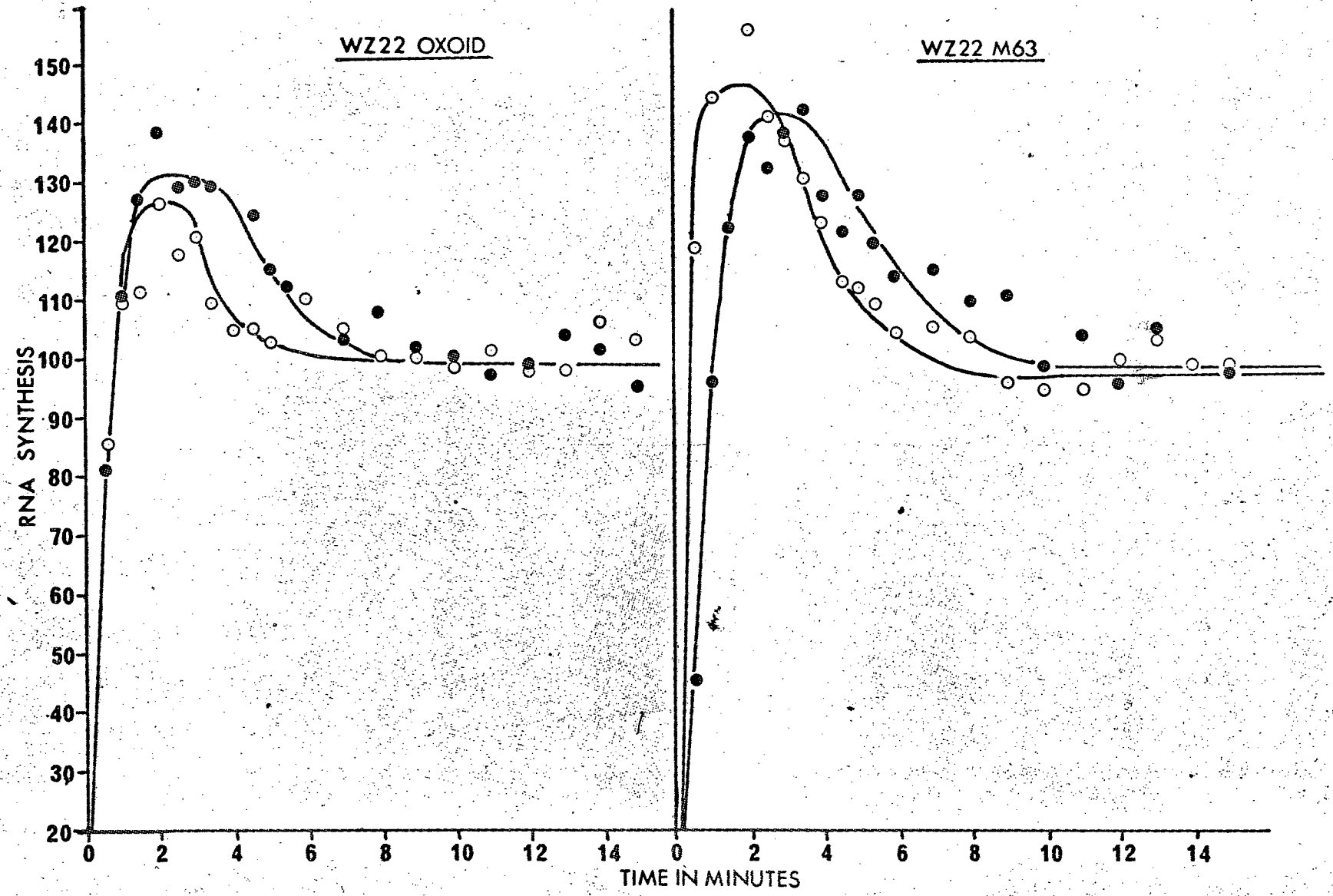
By contrast, in cultures of WZ30 grown in minimal medium and

shifted to 42°C I continue to see a peak of unstable RNA, Figure 4a. The most obvious explanation for these results is that alt-1 is a factor necessary for the synthesis of catabolic enzymes needed to break down the complex nutrients in broth. In minimal medium the majority of enzymes needed will be anabolic and so will be unaffected by alt-1.

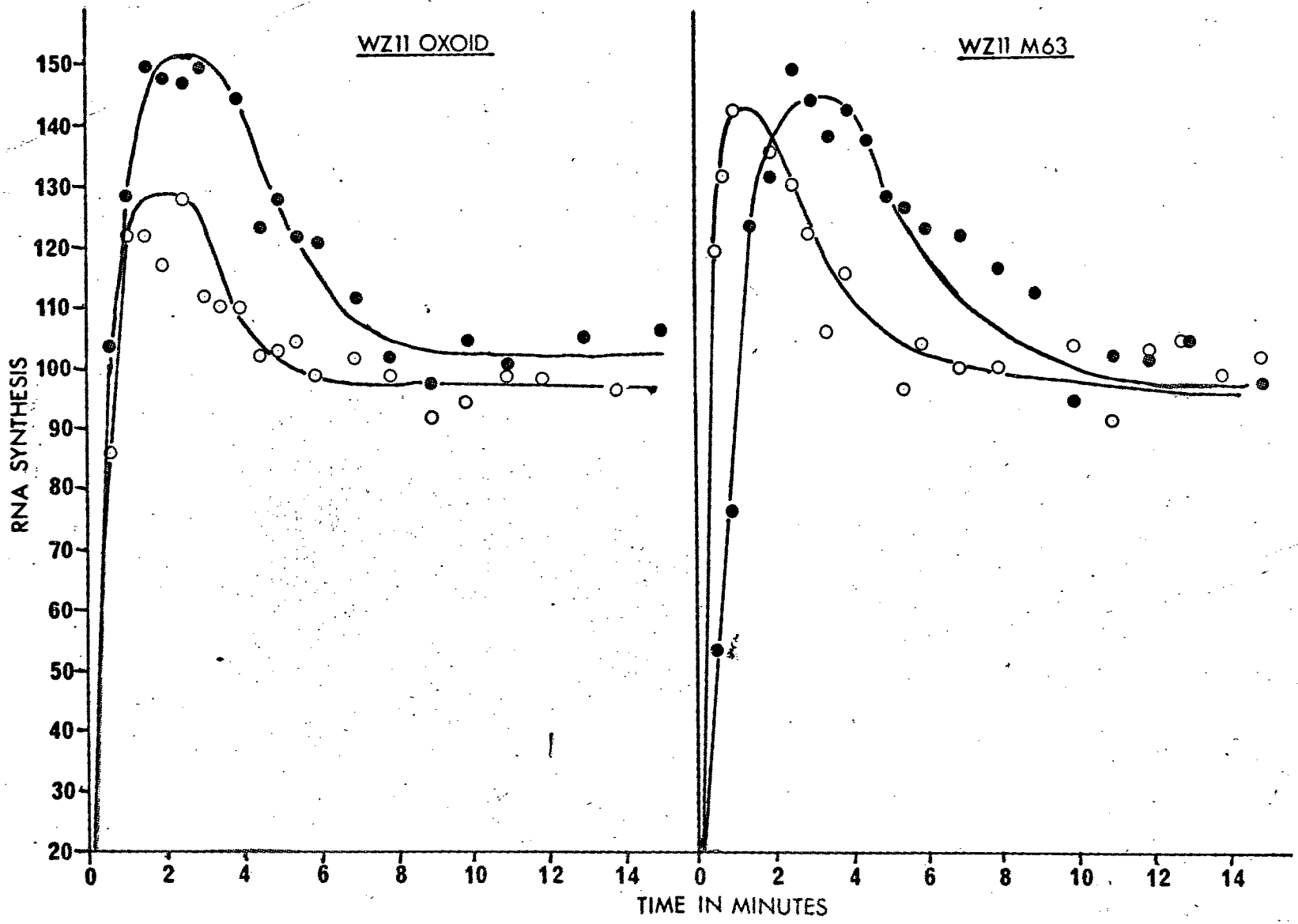
There is a formal possibility the alt-1 product is a nuclease which becomes very active ('wild') at 42°C and rapidly degrades the class of unstable RNA made in broth. I consider this possibility to be unlikely for several reasons. Firstly, heterodiploid alt-1/alt$^+$ strains are temperature-resistant, (section 3,III,b), one would expect a 'wild' nuclease to be dominant not recessive. Secondly, the β-gz level in WZ30 (cya$^-$ alt-1) at 42°C is the same as its parent WZ22 (cya$^-$) at 42°C, that is about 2% of wild type level (section 3,V). One would expect a 'wild' nuclease to reduce the level to zero. Thirdly, WZ57 (cya$^+$ alt-1) shows a β-gz level of about 50% wild type at both 30°C and 42°C (section 3,V), if alt-1 was a 'wild' nuclease, one would expect it to be independent of the cya allele and β-gz levels in WZ57 at 42°C to be near zero.


WZ30 OXOID
WZ30 M63
RNA SYNTHESIS
150
140
130
120
110
100
90
80
70
60
50
40
30
20
0
2
4
6
8
10
12
14
TIME IN MINUTES

4a


WZ22 OXOID
WZ22 M63
RNA SYNTHESIS
150
140
130
120
110
100
90
80
70
60
50
40
30
20
0
2
4
6
8
10
12
14
TIME IN MINUTES

L4b


WZ11 OXOID
WZ11 M63
RNA SYNTHESIS
150
140
130
120
110
100
90
80
70
60
50
40
30
20
0
2
4
6
8
10
12
14
TIME IN MINUTES

4c

Legend to figures 4 a,b,c.

The alt-1 product affects unstable RNA synthesis

The experiments were performed as described in methods (section 2,II,g). Results are expressed as a percentage of the average plateau value for the experiment against time of sampling after addition of ^{3}H uridine rifampicin mixture.

In absolute terms the counts obtained at 42° were in all cases higher than those obtained at 30°. For several reasons it was considered unadvisable to compare the absolute numbers. The cell density of the cultures when labelled at 42° was higher than those used at 30° and so more labelled precursor would be incorporated per unit time. Because more of the cold uridine would have been incorporated before sampling at 42° than when sampling at 30°, the specific activity of the uridine pool would be higher by an unknown factor. As the growth rate is higher at 42° than at 30°, the rate of incorporation would be higher at 42° than at 30°.

d. The effect of rel status on alt-1 suppression

Recent reports on a factor ψr involved in the transcription of ribosomal RNA cistrons (Travers, Kamen, Scheif, 1970) (section 1,VII, f), and the involvement of a small nucleotide guanosine tetraphosphate (ppGpp) possibly analogous to c-AMP in the function of the rel gene (Cashel, Gallant, 1969) suggested that an altered ψr factor may be responsible for the alt-1 suppression. W1655 (metB$^-$) called WZ11 in the strain list, from which the alt-1 strains are derived is rel$^-$ (ribosomal RNA synthesis is not stringently controlled) and it was therefore decided to construct rel$^+$ derivatives of WZ30 to see if this had any affect on β-galactosidase synthesis or the temperature sensitivity of alt-1.

As can be seen from Table 4 that all four rel$^+$ transductants were still temperature sensitive when streaked onto oxoid agar. The rel genotype did not affect the levels of β-galactosidase as all twenty transductants tested whether rel$^+$ or rel$^-$ gave enzyme levels comparible to WZ30 (cya$^-$ alt-1 rel$^-$). The possibility of ψr being involved in alt-1 suppression is therefore unlikely although final proof of showing no change in the kinetics of RNA synthesis has not been obtained.

TABLE 4

The Effect of rel^+ on alt-1

	Number of transductants tested.	Number rel^+	Number of rel^+ still TS^- (alt-1)
P1 lysate from rel^+ strain	10	4	4
P1 lysate from rel^- strain	10	0	-

The assays for rel status were done as described in methods (section 2,II,h).

The transduction of the rel locus was performed as described in strain construction (section 2,III).

V. Suppression by the alt-1 mutation is temperature-dependent

In the preceding section, I have presented evidence that in strains with the alt-1 mutation the synthesis of a class of unstable RNA molecules is temperature sensitive. This property I attribute to the alt-1 mutation itself since in the alt$^+$ parent RNA synthesis is not temperature-sensitive. Can the ability of a cya$^-$ alt-1 strain to grow on lactose and arabinose also be attributed to the alt-1 mutation? Two lines of evidence support this conclusion. Firstly, temperature-resistant revertants (660/660) and recombinants (69/69) of WZ30 concomitantly become Ara$^-$. Secondly, I can show that the increased potential for β-gz and arabinose isomerase synthesis in cya$^-$ alt-1 strains is observed only at the permissive temperature, implying that synthesis of these enzymes is mediated by a heat labile (alt-1) protein. As can be seen from table 5, alt-1 suppresses the cya$^-$ mutation in WZ30 about five-fold raising the level from around 3% to about 15% of wild type. The suppression by alt-1 is temperature sensitive, the level of β-gz in WZ30 at 42°C is reduced to that of WZ22 the parent (Table 5 and Figure 3a).

The results of the β-gz assays in the presence of 10^{-3}M cAMP show some unexpected traits (Table 5). cAMP only partly restores the level of β-gz with WZ30, one would expect the restoration of a normal crp-cAMP system to fully restore β-gz levels as with WZ22. This suggests that alt-1 in some way interferes with the normal transcription process. This view is confirmed with WZ57 (cya$^+$ alt-1), where restoration of a normal crp-cAMP system raised β-gz levels to only 50% of wild type levels. One would expect this interference to be removed when the assay is done at 42°C with WZ57 but this does not

happen. It could be possible that a stable temperature resistant complex is formed by the crp cAMP alt-1 systems.

a. Is the alt-1 protein a transcription factor.

Suppression by alt-1 could occur in at least two ways. We could suppose that the alt gene product is directly involved in the transcription process. Thus when an alt-1 mutant growing at 30°C is raised to 42°C the mutant protein is inactivated and transcription stops. If this is true thenone would expect RNA synthesis to respond rapidly to the shift-up at a rate determined by the rate of alt-1 protein inactivation. Alternatively, the alt-1 gene product could be an enzyme responsible for the production of a small effector molecule analogous to cAMP. In this case one would expect the response to temperature to be slower since it would depend on the dilution of the effector molecule during growth and cell division. To distinguish these hypotheses, I measured the rate at which β-gz synthesis changes during a shift from 30°C to 42°C (Figures 5a, 5b).

The rate of production of β-gz in WZ22 at 30° and 42°C are the same and at a low level, this is expected as WZ22 is the same as WZ11 except for the cya⁻ marker. Strain WZ30 shows a rate of enzyme production five-fold higher at 30° than its parent WZ22. However, after switching to 42°C there is a rapid fall in the rate of enzyme production to a level comparible with WZ22. Note that β-gz synthesis in WZ22 and WZ11, its wild type parent, are not affected by temperature shift (Figures 5a, 5b). The fall in the rate of β-gz production by WZ30 is very rapid being complete in 15 minutes (long before the cell mass has doubled (Figure 5a)). Thus

TABLE 5

β-galactosidase levels and their response to cAMP and temperature in cya⁻ crp⁻ and alt-1 strains.

Strain	Relevant Genotype	30°- cAMP	30°+ cAMP	42°- cAMP
WZ11	cya⁺ alt⁺	100	100	100
WZ22	cya⁻ alt⁺	3.2	103	3.3
WZ25	crp⁻ alt⁺	2.7	2.6	3.0
WZ30	cya⁻ alt-1	15	33	3.2
WZ57	cya⁺ alt-1	47	48	46

Results are expressed as a percentage of wild type levels under the conditions of the assay.

Assays were performed as described in materials and methods (section 2,II,e), c-AMP was added to a final concentration of 10^{-3} M.

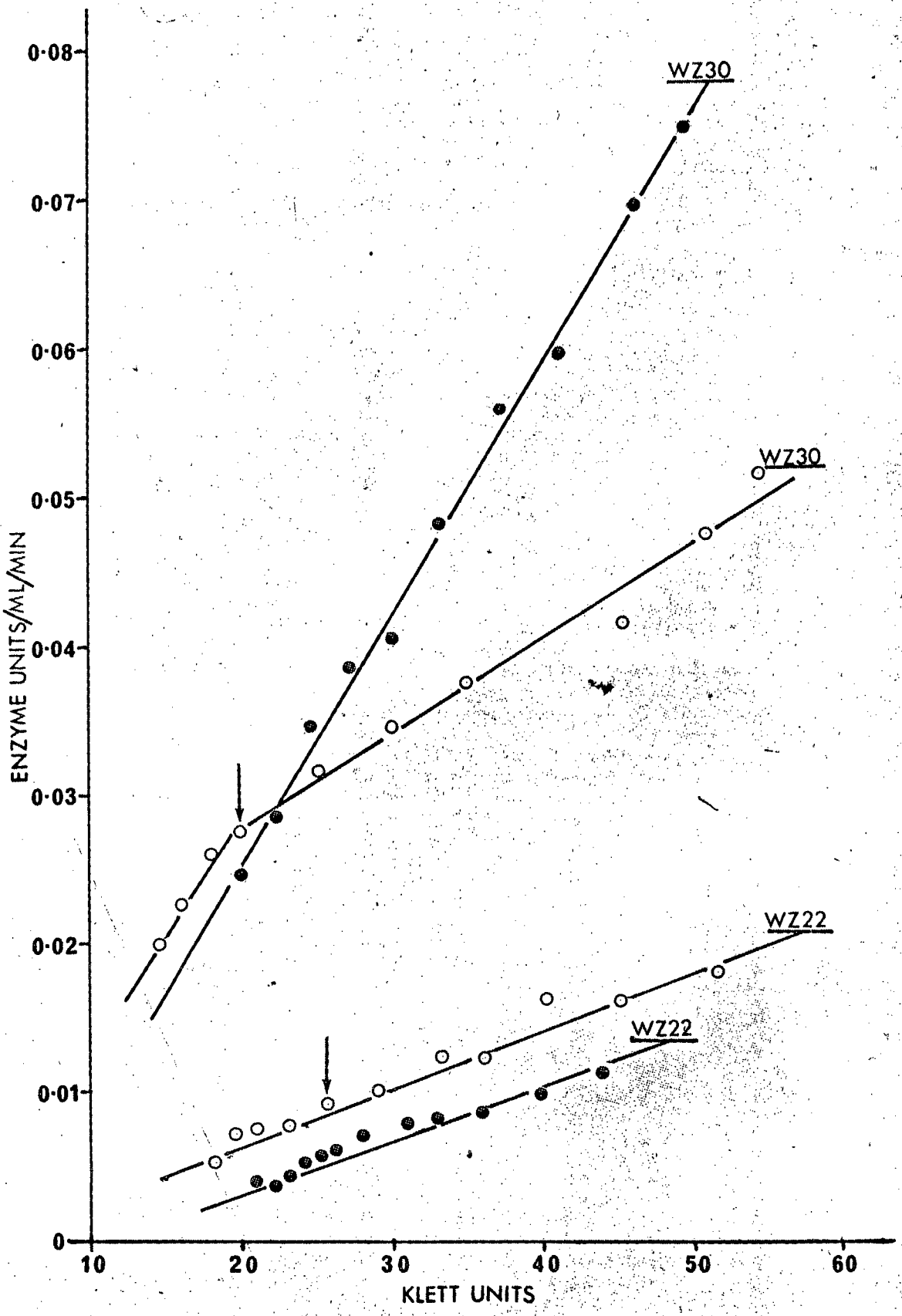
The wild type strain WZ11 produces almost twice as much β-galactosidase in the presence of cAMP due to relief of catabolite repression in the glucose grown cultures. It was not possible to do the assays on glycerol grown cultures as WZ22 and WZ25 do not grow on glycerol.

we favour the view that the alt-1 gene product is a protein directly involved in transcription. If the alt-1 gene product produced an effector molecule one would predict a much slower change in the rate of enzyme synthesis reflecting the dilution of the effector.

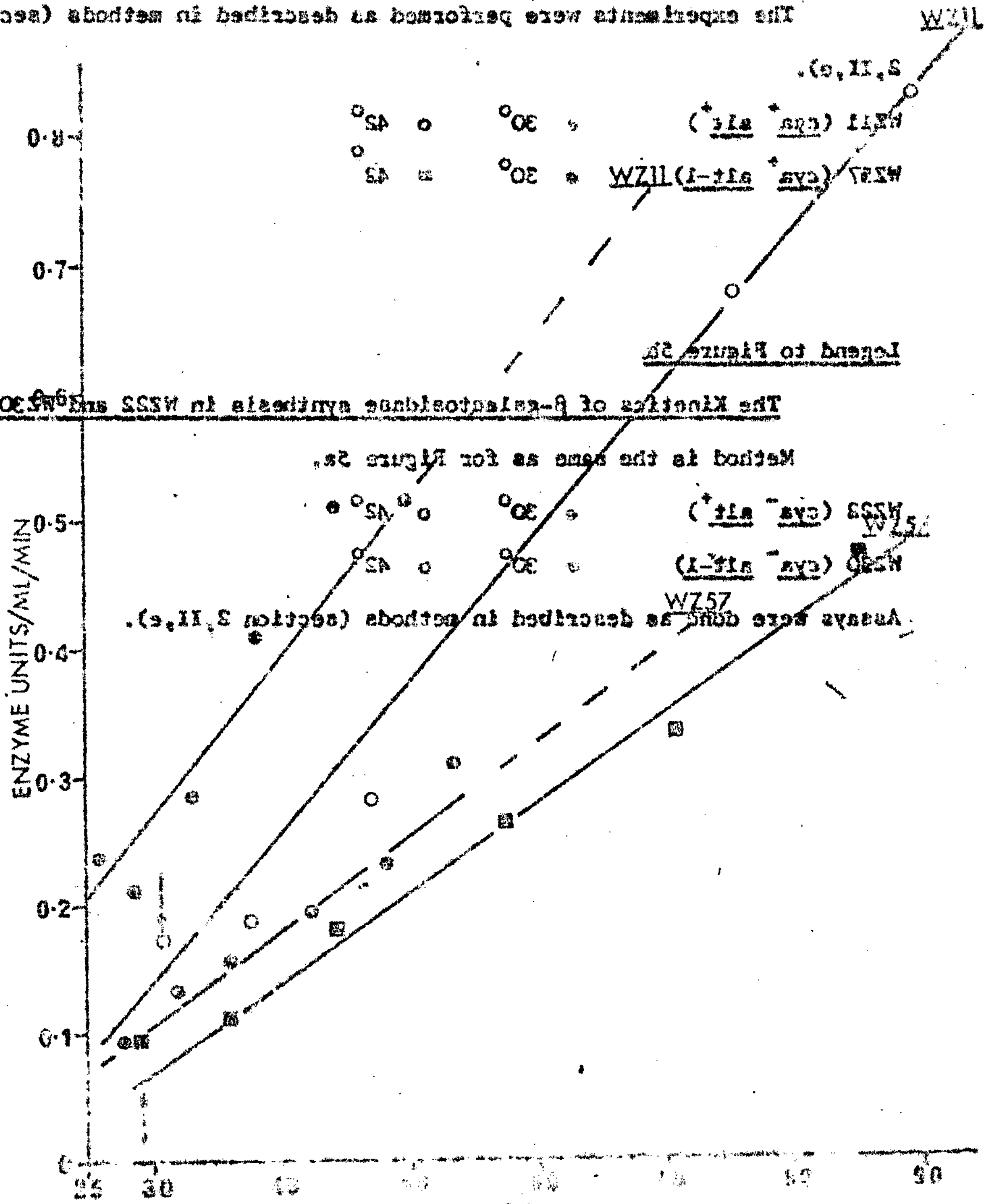
From these results it was concluded that alt-1 is probably a protein which is rapidly inactivated at 42°C. It has a function which is vital to the cell as the strain is temperature-sensitive for growth on both rich and minimal media. The temperature-sensitive form of the alt protein is used by catabolic systems as growth stops much more rapidly in rich medium than minimal. No prediction can be made about its essential functions. The altered form can be used to maintain growth for a considerable period after it has ceased to be used for catabolic systems, but this activity is not enough to maintain growth indefinitely.

VI. alt 1 should suppress not only cya⁻ but also crp⁻.

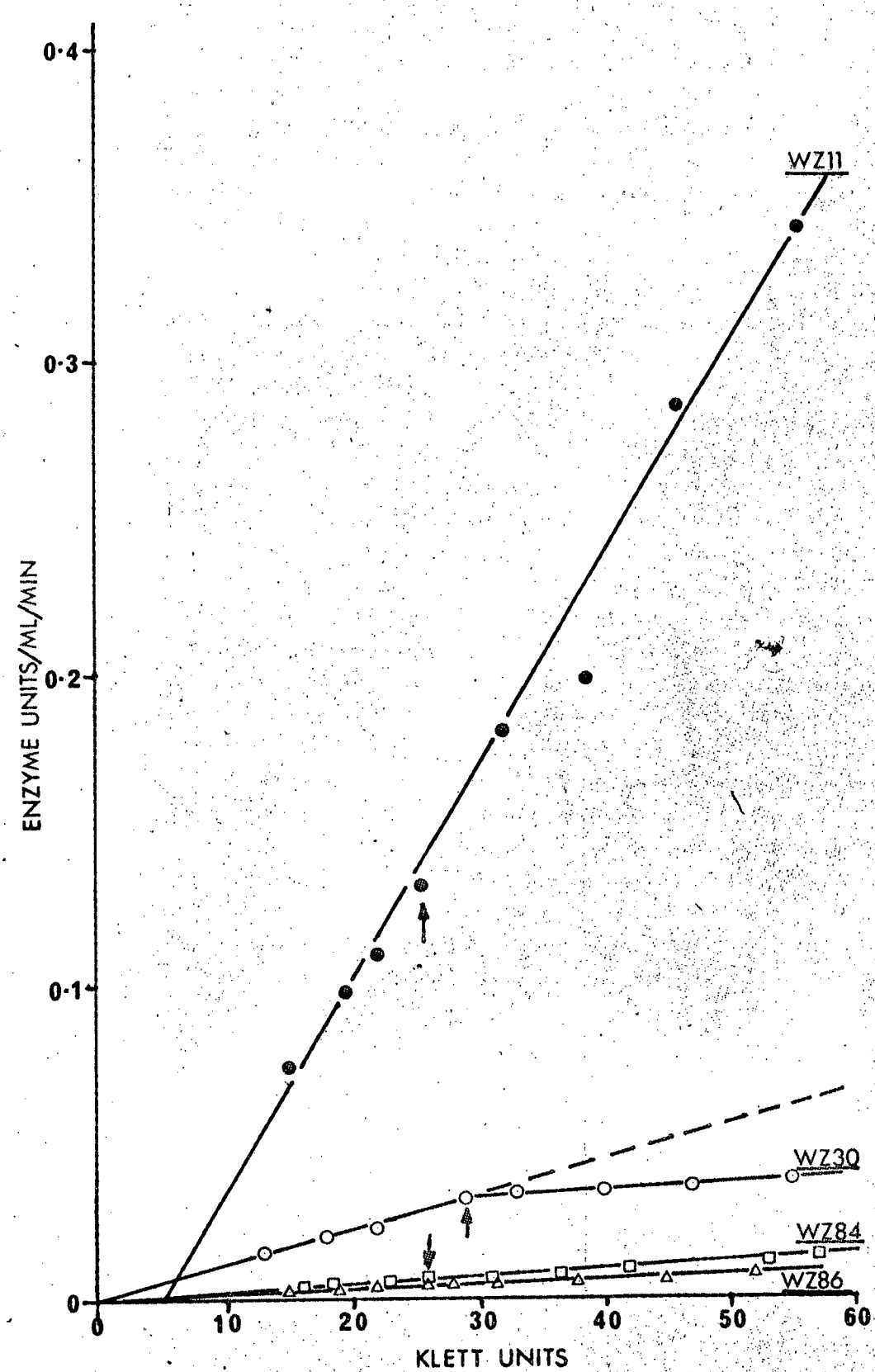
If alt-1 is a protein factor which can substitute for the crp cAMP system in the transcription of catabolite sensitive operons it should suppress a crp⁻ and a crp⁻ cya⁻ strain as well as a cya⁻ strain. The construction of WZ84 (crp⁻ cya⁻ alt-1) and WZ86 (crp⁻ alt-1) is described in methods (section 2,III). During the construction of these strains it was observed that while WZ84 and WZ86 grew on minimal arabinose at 30°C, they did not grow on minimal lactose, xylose or glycerol. This observation led to a study of the synthesis of the β-gz and arabinose-isomerase enzymes in these strains.


0·08
0·07
0·06
0·05
0·04
0·03
0·02
0·01
0
ENZYME UNITS/ML/MIN
WZ30
WZ30
WZ22
WZ22
10
20
30
40
50
60
KLETT UNITS

5a



5b


0·4
0·3
0·2
0·1
0
ENZYME UNITS/ML/MIN
WZ11
WZ30
WZ84
WZ86
0
10
20
30
40
50
60
KLETT UNITS

Legend to Figure 6a

The Kinetics of β-galactosidase synthesis in WZ11, WZ30, WZ84 and WZ86

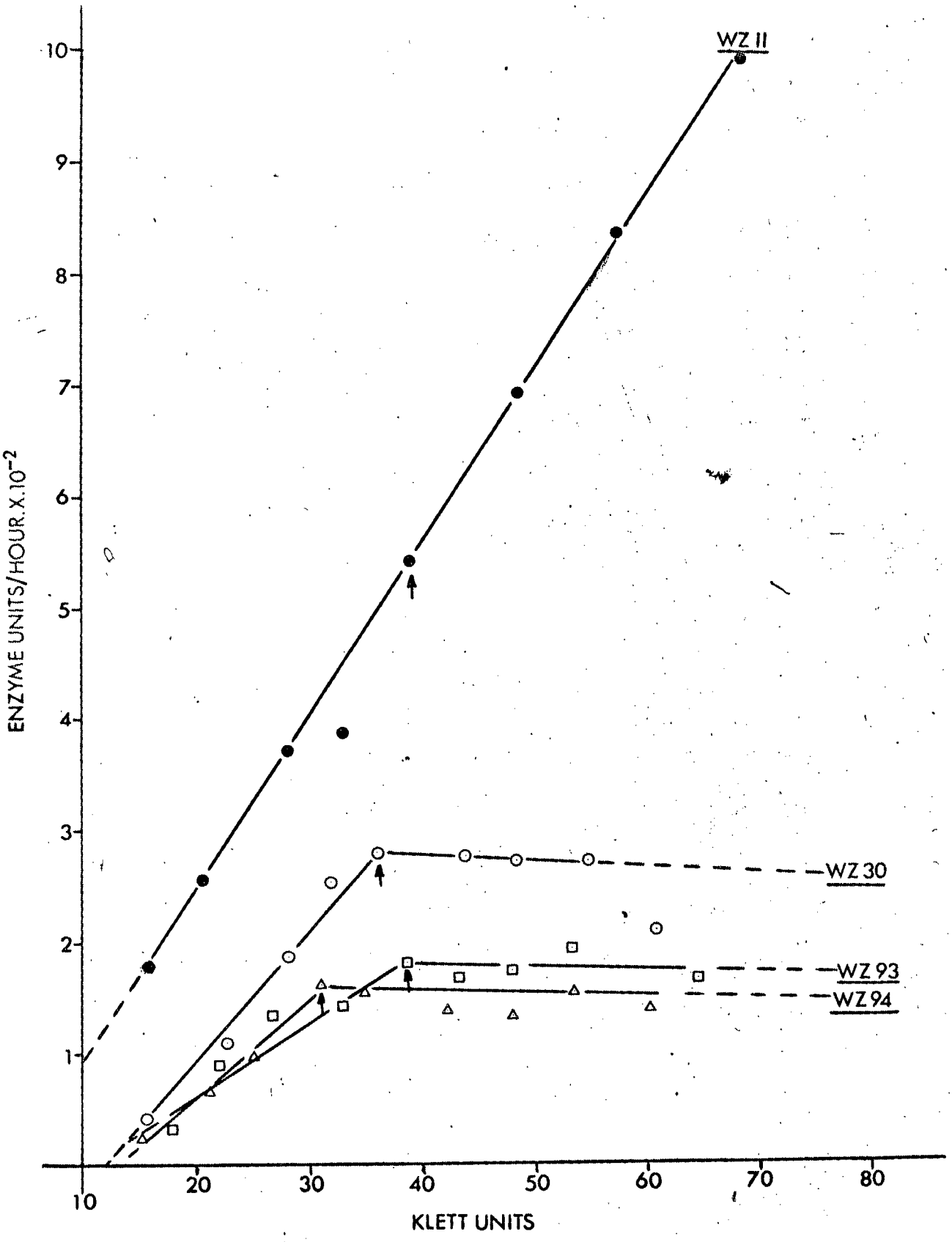
β-galactosidase assays were done as described in methods (section 2,IIe). At points indicated by arrows cultures were switched to 42°.

WZ11 - *cya*$^+$ *crp*$^+$ *alt*$^+$

WZ30 - *cya*$^-$ *crp*$^+$ *alt-1*

WZ84 - *cya*$^-$ *crp*$^-$ *alt-1*

WZ86 - *cya*$^+$ *crp*$^-$ *alt-1*


WZ 11
WZ 30
WZ 93
WZ 94
ENZYME UNITS/HOUR.X.10⁻²
KLETT UNITS
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80

Legend to Figure 6b

Kinetics of Arabinose Isomerase Synthesis in WZ11, WZ30, WZ93 and WZ94

Assays were performed as described in methods (section 2,II,f). With WZ11 the first three samples were incubated with assay mix for 15 minutes, the second three for 30 minutes, all other samples were incubated for 60 minutes.

Cultures were switched from 30° to 42° at points indicated by arrows.

WZ11 - *cya*$^+$ *crp*$^+$ *alt*$^+$

WZ30 - *cya*$^-$ *crp*$^+$ *alt-1*

WZ93 - *cya*$^-$ *crp*$^-$ *alt-1* *Gly*$^+$ (from WZ84)

WZ94 - *cya*$^+$ *crp*$^-$ *alt-1* *Gly*$^+$ (from WZ86)

Preliminary assays with WZ84 WZ86 on glucose or gluconate grown cultures showed that catabolite repression under these conditions made it impossible to measure enzyme levels by the method used. It was therefore necessary to select revertants of WZ84 and WZ86 that would grow on glycerol but not lactose or xylose (see section 2,III).

TABLE 6

β-gz and arabinose-isomerase levels in alt-1 strains

Strain	Genotype	β-gz		Arabinose-isomerase	
		30°	42°	30°	42°
WZ11	*cya*$^+$ *crp*$^+$ *alt*$^+$	100	100	100	100
WZ30	*cya*$^-$ *crp*$^+$ *alt-1*	19	3	80	0
WZ84	*cya*$^-$ *crp*$^-$ *alt-1*	4	4	-	-
WZ86	*cya*$^+$ *crp*$^-$ *alt-1*	2	2	-	-
WZ93	WZ84 *gly*$^+$	-	-	46	0
WZ94	WZ86 *gly*$^+$	-	-	56	0

Results are expressed as a percentage of wild type levels and are calculated from the slopes of Figures 6a and 6b.

Comparing *ara* and *lac* operon expression in these strains I confirmed that suppression to Lac$^+$ by *alt-1* only occurs in the cya$^-$ strain, while suppression to Ara$^+$ occurs in the *cya*$^-$, *cya*$^-$*crp*$^-$ and *crp*$^-$ strains (Figures 6a, 6b, Table 6). It would seem that *alt-1* is distinguishing a radical difference between *lac* and *ara* expression. The most obvious difference between these two operons is that *lac* is primarily negatively controlled while *ara* is positively controlled. The difference in suppression between the *lac* and *ara* operons by *alt-1* may therefore be reflecting promoter structure.

PART 4

DISCUSSION

The selection procedure used appears to be very good for obtaining mutations affecting RNA synthesis. Temperature-sensitive mutations in the *alt* region occur at a high frequency. Seventy four independent temperature sensitive pleiotropic Ara$^+$ revertants from *cya*$^-$ and *crp*$^-$ parents all mapped in the same region as *alt-1* (Silverstone, Goman, Scaife, 1972). Although only one of these has been studied extensively it is reasonable to suppose that some of the other mutations are of the *alt* type.

The possibility of a second mutation not in the *alt* region being necessary for suppression of the *cya*$^-$ and *crp*$^-$ mutations has been raised by the inability of a KLP2 *alt-1* episome to suppress a *cya*$^-$ *crp*$^-$ strain. Preliminary results suggest that this second gene may be the gene coding for the β subunit of RNA polymerase as defined by *rif*^{r} mutations. It seems likely that strain differences in the β subunit gene and not an alteration in this gene during selection of the *alt-1* strain (WZ30) are responsible for this failure to get suppression with the *alt-1* episome. Strain WZ75 which was the strain I attempted to suppress with the *alt-1* episome is derived from JC411 whereas the *alt-1* strain is derived from W1655. Martin Rabstein (personal communication) has been able to transduce, in one step, a derivative of WZ22 (*cya*$^-$) to Ara$^+$ TS$^-$ with a P1 lysate from WZ30 (*cya*$^-$ *alt-1*), showing that the *Alt-1* phenotype is a mutation(s) in a small region of the chromosome transducible with P1.

It is possible that the second site muation occured during the

UV mutagenesis of WZ11 in the isolation of WZ22 (section 2,III). This possibility could be confirmed or eliminated by constructing a *crp*$^-$ derivative of WZ11, by P1 transduction, and then looking to see if the Ara$^+$ TS$^-$ phenotype can be obtained by a single P1 transduction using a lysate from WZ30.

Should the ability of *alt-1* to suppress prove to depend on the particular β subunit gene present in the strain interesting speculations about the identity of the *alt-1* gene product and its mode of action in relation to RNA polymerase are raised. The *alt* gene product could be a subunit of the RNA polymerase core enzyme, the sigma factor, or a sigma like factor. The results of the RNA pulse-labelling experiments show that *alt-1* does confer specificity on the transcription it stimulates in that unstable RNA synthesised in rich medium is the class most dramatically affected by a switch to high temperature. Whether *alt-1* acts by directly conferring on the polymerase a capacity for recognising certain promoters or by interacting with certain promoters so that they are more easily recognised by polymerase is not known from results so far. Investigation of this question will probably involve setting up an *in vitro* transcription system.

The formal possiblity still exists that the suppression by *alt-1* is mediated through a small effector molecule. This is unlikely unless there is a very rapid turnover of the effector or if the effector can only be used a very limited number of times. The temperature sensitivity of the *alt-1* function and the rapidity with which the shift to high temperature affects the production of β-gz and arabinose-isomerase strongly suggest that *alt-1* is a protein directly

involved in transcription.

The different suppression patterns shown with β-gz and arabinose-isomerase can be explained by invoking the positive role played by the ara C protein. The alt-1 proteins may interact with the ara C protein, under inducing conditions, to promote transcription and completely circumvent the need for the crpcAMP complex. The lac transcription system does not have such a protein and it may be that the expression of the lac genes in alt-1 strains requires the crp protein. This view is supported to some extent by the work of Lang-Yang and Zubay (1973) in which they show, in an in vitro system, that the addition of purified ara C protein stimulates transcription of the ara operon and removes the requirement for the crp-cAMP system. Beckwith (personal communication) has isolated Ara^+ revertants of a cya^- strain and found that some of these mutations map in the ara C gene. This shows that the requirement for the crpcAMP system in transcription can be removed by an alteration in the ara C protein. The validity of this explanation for the different suppression could best be answered by constructing an ara C deletion, crp^- alt-1 strain and looking at the effect on arabinose isomerase production.

The difference in suppression patterns may also reflect a basic difference in the promoters of positively (ara mal) and negatively (lac)controlled operons. This model could be investigated by looking at alt-1 suppression in another positively controlled operon such as maltose.

The formal possibility that the cya^- mutation used being leaky and producing low levels of cAMP still exists. This low level of

cAMP may be enough to permit expression of the *ara* operon but not of the *lac* operon in *alt-1* strains. This does not seem likely as one would expect all catabolite sensitive operons to respond to the same levels of cAMP. The introduction of a *cya* deletion, which makes no cAMP, should provide a method for eliminating this model.

PART 5

REFERENCES

ARDITTI, R.R., SCAIFE, J.G., BECKWITH, J.R. (1968) J. Mol. Biol. 38. 421.

BECKWITH, J.R., GRODZICKER, T., ARDITTI, R.R. (1972). J. Mol. Biol. 69. 155.

BERG, P., BARRETT, K.J., HINKLE, D., McGRATH, J., CHAMBERLIN, M. (1969) Fed. Proc. 28. 659

BERMAN, M., LINN, E.C.C. (1971) J. Bact. 105. 113.

BHORJEE, J.S., FOWLER, A.V., ZABIN, I. (1969) J. Mol. Biol. 43. 219

BLATTNER, F., DHALBERG, J.E. (1972) Nature 237. 227.

BOURGEOIS, S. (1965) J. Mol. Biol. 14. 300.

BOURGEOIS, S., JOBE, A. (1970) The Lactose Operon 325. Eds. Beckwith J.R., ZIPSER, D.

BREMER, H., YUAN, D. (1968) Biochem. Biophys. Acta (Amst) 169. 21

BURGESS, R.R., TRAVERS, A.A. DUNN, J.J., BAUTZ, E.K.F. (1969) Nature 221. 43.

CASHEL, M., GALLANT, J. (1969) Nature 221. 838.

CHEN, B., CROMBRUGGHE, B.de., ANDERSON, W.B., GOTTESMAN, M.E., PASTAN, I. (1971) Nature 233. 67.

CLARK, D.J., MARR, A.G. (1964) Biochem. biophys. Acta (Amst) 92. 85

COHN, M., HORIBATA, K. (1959) J. Bact. 78. 601.

COHN, M., TORRIANI, A.M. (1952) J. Immunolog 69. 471.

CRAVIN, G.R., STEERS, E.J., ANFINSEN, C.B. (1965) J. Biol. Chem. 240. 2468.

CRIBBS, R., ENGELSBERG, E. (1964) Genetics 49. 95

CROMBRUGGHE, B. de, CHEN, B., ANDERSON, W.B., NISSLEY, P., GOTTESMAN, M.E., PASTAN, I. (1971) Nature 231. 139

CROMBRUGGHE, B.de, PERLMAN, R.L., VARMUS, H.E., PASTAN, I. (1969) J. Biol. Chem 244. 5828

DAVIDSON, J., BROOKMAN, K., PILARSKI, L.M., ECHOLS, H. (1970) Cold Spr. Harb. Symp. Quant. Biol. 35. 95.

DAVIDSON, J., PILARSKI, L.M., ECHOLS, H. (1969) Proc. Nat. Acad. Sci. (Wash) 63. 168

DISCHE, Z. (BORENFREUND (1951) J. Biol. Chem. 192. 583.

EMMER, M., CROMBRUGGHE, B. de, PASTAN, I., PERLMAN, R. (1970) Proc. Nat. Acad. Sci. (Wash) 66. 480.

ENGELSBERG, E., IRR, J., POWER, J., LEE, N. (1965) J. Bact. 90. 946

ENGELSBERG, E., SHEPPARD, D., SQUIRES, C., MERONK, F.Jr. (1969) J. Mol. Biol. 43. 281.

ENGELSBERG, E., SQUIRES, C., MERONK, F.Jr. (1969) Proc. Nat. Acad. Sci. (Wash) 62. 1100.

EPSTEIN, W., NAONO, S., GROS, F. (1966) Biochem. biophys. Res. Commun. 24. 588

ERON, L., BLOCK, R. (1971) Proc. Nat. Acad. Sci. (Wash) 68. 1828

FIIL, N. (1969) J. Mol. Biol. 45. 195.

FOX, C.F. (1966) J. Mol. Biol. 19. 576.

FOX, C.F., CARTER, J.R., KENNEDY, E.P. (1967). Proc. Nat. Acad. Sci (Wash) 57. 698.

GILBERT, W. (1967) Proc. Nat. Acad. Sci. (Wash) 58. 2415

GILBERT, W., MULLER-HILL, B. (1966) Proc. Nat. Acad. Sci. (Wash) 56. 1891.

GROSS, J.D., ENGELSBERG, E. (1959) Virology 9, 314

GUTHRIE, P., PARDEE, A.B. (1969) J. Gen. Physiol. 54, 93

HAZELTINE, W.A. (1972) Nature 235, 329

HEIL, A., ZILLIG, W. (1970) Febs Letters 11, 165

HORIUCHI, T., YASUMI, O. (1966) J. Mol. Biol. 20, 517

IPPEN, K., MILLER, J.H., SCAIFE, J.G., BECKWITH, J.R. (1968) Nature 217, 825.

JACOB, F., MONOD, J. (1961) Cold Spr. Harb. Symp. Quant. Biol. 26, 193

JOBE, A., BOURGEOIS, S. (1972a) J. Mol. Biol. 69, 397

JOBE, A., BOURGEOIS, S. (1972b) J. Mol. Biol. 72, 139

JOBE, A., RIGGS, A.D., BOURGEOIS, S. (1971) J. Mol. Biol. 64, 181

JONES, T.H.D., KENNEDY, E.P. (1969) J. Biol. Chem. 244, 5981

KABACK, H.R. (1969) Proc. Nat. Acad. Sci. (Wash) 63, 724.

KEPES, A. (1960) Biochem. biophys. Acta (Amst) 40, 70

KUNDIG, W., GHOSH, S, ROSEMAN, S. (1964) Proc. Nat. Acad. Sci. (Wash) 52, 1067

LANG-YANG, H., ZUBAY, G. (1973) Molec. gen. Genet. 122, 731

LEDERBERG, E., LEDERBERG, J. (1953) Genetics 38, 51

LIN, S., RIGGS, A.D., (1972) J. Mol. Biol. 72, 671

LOSICK, (1972) Ann. Rev. Biochem. 41, 409

LOW, B., (1968) Proc. Nat. Acad. Sci. (Wash) 60, 160.

LOWRY, O.H., ROSEBROUGH, J., FARR, ALL., RANDALL, R.J. (1951) J. Biol. Chem. 193, 265.

MASTERS, M., BRODA, P.M.A. (1971) Nature 232, 137

MATNEY, T.S., GOLDSCHMIDT, EPP., ERWIN, M.S. (1963) Biochem. Biophys. Res. Commun. 12, 483

MILLER, J.H., IPPEN, K., SCAIFE, J.G., BECKWITH, J.R. (1968) J. Mol. Biol. 38, 413.

MILLER, J.H., MULLER-HILL, B., BECKWITH, J.R. (1968) Nature 220, 1257

MILLER, J.H., REZNIKOFF, W.S., SILVERSTONE, A.E., IPPEN, K., SIGNER, E.R., BECKWITH, J.R. (1970) J. Bact. 104, 1273

MULLER-HILL, B. (1966) J. Mol. Biol. 15, 374

MULLER-HILL, B., CRAPO, L., GILBERT, W. (1968) Proc. Nat. Acad. Sci. (Wash) 59, 1259

NAKANISHI, S., ADHYA, S., GOTTESMAN, M.E., PASTAN, I. (1973) Proc. Nat. Acad. Sci. (Wash) 70, 334

ODA, T., TAKANAMI, M. (1972) J. Mol. Biol. 71, 799

PARDEE, A.B., JACOB, F., MONOD, J. (1959) J. Mol. Biol. 1, 165

PASTAN, I., PERLMAN, R.L. (1969) J. Biol. Chem. 244, 2226

PASTAN, I., PERLMAN, R.L. (1969) J. Biol. Chem. 244, 2836

PATO, M., MEYENBERG, K. VON, (1970) Cold. Spr. Har. Symp. Quant. Biol. 35, 497

PERLMAN, R., CROMBRUGGHE, B. de., PASTAN, I. (1969) Nature 223, 810.

PERLMAN, R., PASTAN, I. (1968) Biochem. biophys. Res. Commun. 30, 656.

PERLMAN, R., PASTAN, I. (1969) Biochem. biophys. Res. Commun. 37, 151

PETTIJOHN, D.E. (1972) Nature 235, 204

PFAHL, M. (1972) Genetics 72, 393

PRESTIDGE, L.S., PARDEE, A.B., (1965) Biochem. biophys. Acta. (Amst.) 100, 591.

REZNIKOFF, W.S., BECKWITH, J.R. (1969) J. Mol. Biol. 43, 215

REZNIKOFF, W.S., MILLER, J.H., SCAIFE, J.G., BECKWITH, J.R. (1969) J. Mol. Biol. 43, 201.

REVEL, H., LURIA, S., ROTMAN, B. (1961) Proc. Nat. Acad. Sci. (Wash) 47, 1956

RICKENBERG, S.V., COHEN, G.N., BUTTIN, G., MONOD, J. (1956) Annls. Inst. Pasteur (Paris) 91, 829

RIGGS, A.D., BOURGEOIS, S. (1968) J. Mol. Biol. 34, 361, 365

RIGGS, A.D., BOURGEOIS, S., NEWBY, R.F., COHN, M., (1968) J. Mol. Biol. 34, 368

RIGGS, A.D., REINESS, G., ZUBAY, G. (1971) Proc. Nat. Acad. Sci. (Wash) 68, 1222

RIGGS, A.D., SUZUCI, H., BOURGEOIS, S. (1970) J. Mol. Biol. 48, 67

ROBERTS, J.W., (1969) Nature 224, 1168.

ROTMAN, B., CELADA, F. (1968) Proc. Nat. Acad. Sci. (Wash) 60, 660

SADLER, J.R., SMITH, T.F. (1971) J. Mol. Biol. 62, 139

SANDERS, R., McGEOCH, D. (1973) Proc. Nat. Acad. Sci. (Wash) 70, 1017

SCAIFE, J.G., BECKWITH, J.R. (1966) Cold. Spr. Harb. Symp. Quant. Biol. 31, 403

SHEPPARD, D.E., ENGELSBERG, E. (1967) J. Mol. Biol. 25, 443

SILVERSTONE, A.E., ARDITTI, R.R., MAGASANIK, B. (1970) Proc. Nat. Acad. Sci. (Wash) 66, 773

SILVERSTONE, A.E., GOMAN, SCAIFE, J.G. (1972) Molec. gen. Genet. 118, 223.

SILVERSTONE, A.E., MAGASANIK, B., REZNIKOFF, W.S., MILLER, J.H. BECKWITH, J.R. (1969) Nature 221, 1012

SMITH, T.F., SADLER, J.R. (1971) J. Mol. Biol. 59, 273

STONINGTON, O.G., PETTIJOHN, D.E. (1971) Proc. Nat. Acad. Sci. Wash) 68, 6

TAYLOR, A.L., TROTTER, C.D. (1972) Bact. Rev. 36, 504

TRAVERS, A.A., BURGESS, R.R. (1970) Nature 222, 537

TRAVERS, A.A., KAMEN, R.I., SCHEIF, R.F. (1970) Nature 228, 748

TYLER, B., MAGASANIK, B. (1969) J. Bact. 97, 550

VOGEL, H.J., BONNER, D.M. (1956) J. Biol. Chem. 218, 97

WALTER, G., ZILLIG, W., PALM, P., FUCHS, S. (1967) Eur. J. Biochem. 3, 194

WILLSON, C., PERRIN, D., COHN, M., JACOB, F., MONOD, J. (1964) J. Mol. Biol. 8, 582

YUDKIN, M.D. (1969) Biochem. J. 114, 307

YUDKIN, M.D. (1971) Biochem. J. 123, 579

ZUBAY, G., GIELOW, L., ENGELSBERG, E. (1971) Nature 233, 164

ZUBAY, G., SCHWARTZ, D., BECKWITH, J.R. (1970) Proc. Nat. Acad. Sci. (Wash) 66, 104

ZABIN, I., KEPES, A., MONOD, J. (1962) J. biol. chem. 237, 253

ACKNOWLEDGEMENTS

I wish to thank, Bill Hayes and the M.R.C. for allowing me to do this project, Dr John Scaife for being a helpful and patient supervisor, and Ivor Titawella for shaming me into actually getting this thesis written.

APPENDIX

ALT: A new factor involved in the synthesis of RNA by *Escherichia coli*.

Silverstone, Goman and Scaife

Molec. gen. Genet. 118, 223—234 (1972)

ALT: A New Factor Involved in the Synthesis of RNA by *Escherichia coli*

A. E. Silverstone*, M. Goman, and J. G. Scaife

MRC Molecular Genetics Unit, Department of Molecular Biology, University of Edinburgh

Received June 20, 1972

Summary. We have defined a new gene, *alt*, which affects RNA synthesis in *Escherichia coli*. Mutants for *alt* arise among revertants of strains lacking the CRP-cAMP system necessary for full expression of catabolite-sensitive operons. Studies on a temperature-sensitive *alt* mutant indicate that the *alt* gene product is necessary for the synthesis of an important class of messenger RNA molecules.

Introduction

The *lac* and *ara* operons of *Escherichia coli*, like other catabolite-sensitive (CS) operons, normally require for their transcription, adenosine-3′,5′-cyclic monophosphate (cAMP), and at least one protein factor, variously termed CAP, CRP, and CGA (Perlman and Pastan, 1968; Ullman and Monod, 1968; Zubay, Schwartz and Beckwith, 1970; Emmer, de Crombrugghe, Pastan and Perlman, 1970; Riggs, Reiness and Zubay, 1971). The CRP-cAMP system is involved in the initiation of *lac* transcription (Magasanik, 1970). Strains whose *lac* expression is no longer dependent on CRP or cAMP have mutations e.g. P^r_{UV5} in the *lac* promoter region (Silverstone, Arditti and Magasanik, 1970; Silverstone, Magasanik, Reznikoff, Miller and Beckwith, 1969). Purified RNA polymerase (holoenzyme) will transcribe *lac* DNA *in vitro* (de Crombrugghe, Chen, Anderson, Nissley, Gottesman, Pastan, and Perlman, 1971; Eron and Block, 1971). Transcription of normal *lac* DNA is dependent on CRP and cAMP whereas transcription of DNA from the promoter mutant P^r_{UV5} is largely independent of these components (Eron and Block, 1971). The purified system therefore mimics *lac* transcription *in vivo* and this suggests that transcription of the *lac* operon involves only three components; RNA polymerase, CRP and cAMP, in addition to the four nucleoside triphosphate substrates of the enzyme. Both biochemical and genetic studies suggest that the *lac* promoter contains at least two sites, one—the target for CRP and the other—the binding site for RNA polymerase (Eron and Block, 1961; de Crombrugghe *et al.*, 1971; Arditti, Grodzicker and Beckwith, in press).

We shall describe a new class of mutation, termed *alt*, which compensates for loss of the CRP-cAMP system. The *alt* mutants could have a changed RNA polymerase able to initiate transcription of CS operons without CRP intervention. Alternatively, they could have an altered transcription factor able to substitute

* *Present address:* Department of Biological Sciences, Schermerhorn Hall, Columbia University, New York, N.Y. 10027, U.S.A.

for the CRP-cAMP system. In its unaltered form, such a factor could normally act in concert with the CRP-cAMP system, being required to initiate transcription of CS operons. In this case, the interpretation of the transcription studies mentioned above, would have to be revised. On the other hand, the factor may normally only be required for transcription of genes of some other class, unrelated to CRP.

Genetic and physiological studies show that *alt* mutations affect transcription of certain operons, suggesting that they alter a transcription factor, rather than the RNA polymerase itself. Our studies favour the conclusion that this factor normally specifies transcription of genes not using CRP.

Materials and Methods

Chemicals. Rifampicin was a gift from Gruppo Lepetit (Milan). Adenine-^{14}C (U) and uridine-5-^{3}H were obtained from Amersham, isopropyl-β-D-thio-galactoside (IPTG) and adenosine-3′,5′-cyclic monophosphoric acid (cAMP) from Sigma Biochemicals, BBOT scintillant from CIBA, and polyethyleneimine cellulose plates (PEI) from Merck (Darmstadt).

Bacterial Strains. E. coli K12 strains used in these experiments are described in Table 1.

Table 1. Bacterial strains

Strains	Pertinent genetic characteristics	Source or derivation
WZ11	F⁻ *metB*⁻ *lac*⁺ *cya*⁺ *crp*⁺ *alt*⁺ *str-r rel*⁻	Streptomycin resistant derivative of W1655. (Lederberg and Lederberg, 1953)
WZ22	F⁻ *metB*⁻ *lac*⁺ *cya*-855 *crp*⁺ *alt*⁺ *str-r rel*⁻	Ultraviolet mutagenesis of WZ11
WZ25	F⁻ *metB*⁻ *lac*⁺ *cya*⁺ *crp*-868 *alt*⁺ *str-r rel*⁻	Ultraviolet mutagenesis of WZ11
WZ30	F⁻ *metB*⁻ *lac*⁺ *cya*-855 *crp*⁺ *alt*-1 *str-r rel*⁻	Pleiotropic, sugar-positive temperature sensitive revertant of WZ22
WZ44	F⁻ *metB*⁻ *lac*⁺ *cya*-855 "*glpK*" *str-r rel*⁻	Glycerol-positive revertant of WZ22. The precise nature of the "glpK" mutation has not been established
WZ57	F⁻ *metB*⁺ *cya*⁺ *crp*⁺ *alt* 1 *str-r*	Met⁺ Lac⁺ recombinant of WZ30 mated with D8007
D8007	Hfr G10, *his*⁻ (Fig. 1)	Matney, Goldschmidt and Erwin (1963)
ED1002	Hfr *lac proB*, integrated F′ts *lac*⁺ *spc-r str-r* (Fig. 1)	Masters and Broda (1971)
ED1009	Hfr *lac proB*, integrated F′ts *lac*⁺ *spc-r str-r* (Fig. 1)	Masters and Broda (1971)
KLF2	F′ *arg*G⁺ *arg*R⁺/*arg*G⁻ *his*⁺ *leu*⁺ *metB recA*⁻ *str*A	Low (4968)

Genetic loci follow standard abbreviations (Taylor, 1970); *cya* is the genetic locus responsible for the production of an ATP-dependent adenyl cyclase (Yokota and Gots, 1970); *crp* is the locus responsible for production of a protein product necessary for transcription of catabolite sensitive operons (Schwartz and Beckwith, 1970; Perlman *et al.*, 1970); *alt* is the locus discussed in this text. Hfr origins and their direction of transfer are indicated in Fig. 1.

Growth Media. Nutrient broth (NB) was Oxoid NB2. Two liquid minimal salts media were used; MS (de Haan and Gross, 1962) and M63 (Pardee, Jacob, and Monod, 1959) to which glucose (0.2%), vitamin B_1 (1 μg/ml), and growth supplements (20 μg/ml) were added. Solid media were obtained by adding Davis New Zealand Agar at 1.25% (NB) or 1.75% (MS) to the appropriate liquid media.

Bacterial Crosses. Our mating methods are described in Scaife and Pekhov (1964).

Assay for β-D-Galactosidase. Exponential cells appropriately diluted in one ml of MS were added at 0° to a mixture of 0.2 ml of 0.1% solution of CeTB (Tyler and Magasanik, 1969), 0.8 ml of PM² buffer (the reducing buffer of Revel, Luria and Rotman, 1961), and 0.01 ml of 1% sodium deoxycholate (DOC). We assayed the samples (Revel *et al.*, 1961) using 1.0 M Na_2CO_3 in 8M urea to terminate the reaction.

Enzyme activity is expressed as the corrected change in OD_{420} per minute per ml of culture.

Measurement of Total RNA Synthesis. Labelling of RNA and inhibition of RNA synthesis is described by Pato and von Meyenberg (1970). Cultures grown in NB or supplemented M63 containing 40 μg/ml uridine were labelled with uridine tritiated at position 5 of the pyrimidine ring (2.5 μCi/ml, 40 μg/ml) to minimise the entry of label into DNA as thymine. Initiation of RNA synthesis was stopped with 200 μg/ml rifampicin. Samples were prepared for counting as described by Bremer and Yuan (1968), and counted in BBOT-toluene (4 g/l).

Cyclic AMP Assay. The assay we used was a combination of methods developed by S. Fogel and by G. Edlin (personal communications). It involves the fractionation, by two-dimensional chromatography, of labelled nucleotides from cells grown in ^{14}C-adenine. The PEI chromatography plates are washed according to Randerath (1966). Cells exponentially growing in supplemented M63 containing 50 μg/ml ^{14}C-Adenine (U) (50 μCi/ml) for 3–4 generations are concentrated on Millipore filters (10^7 cells), and then extracted in 2.5 ml of 0.3M perchloric acid at 0° C. The debris is removed from the extract by centrifugation and the supernatant is neutralized with two drops of EDTA (0.5M) + KOH (6M). The precipitate is removed by centrifugation and 40 μl of the supernatant, adjusted to a standard specific activity is spotted on the PEI cellulose plate with a 10 μl solution of cAMP (10 mM) as a marker. Each sample is fractionated by two dimensional LiCl chromatography (Cashel and Gallant, 1969). The cAMP marker shows up as a fluorescent spot under ultraviolet light. This spot is cut out of the chromatogram and its radioactivity measured in a gas-flow counter (Nuclear Chicago).

Results

The Isolation of Alt Mutations. We have derived *alt* mutants from two different starting strains. One of these is WZ22 which has a mutation, *cya*-855, which reduces the intracellular concentration of cAMP to less than 5 per cent of the normal level (Table 2). The mutation *cya*-855 maps between *metE* and *ilv* (Silverstone, Tsuji and Scaife, unpublished), the region containing the gene for adenyl cyclase (Yokota and Gots, 1970). The expression of catabolite-sensitive operons is much reduced in WZ22 (Table 2), a defect which we attribute to inactivation of the CRP-cAMP system. As expected, the defect can be reversed by exogenous cAMP (Table 2).

The second strain giving rise to *alt* mutants, WZ25, has a different mutation, *crp*-868, also reducing the expression of CS operons. However, the mutation does not affect the cAMP concentration in WZ25, and its effect on CS operons cannot be reversed by exogenous cAMP (Table 2). The mutation therefore has the phenotype expected for a mutant lacking the CRP factor. Strong support for this conclusion comes from our observation that the *lac* promoter mutant, p^r_{UV5}, which is transcribed in the absence of the CRP-cAMP system (Schwartz and Beckwith, 1970), can be transcribed in *crp*-868 (data not shown). As expected,

Table 2. β-galactosidase synthesis and cyclic AMP levels in wild type and mutant strains

Strain	Relevant genotype	β-D-Galactosidase synthesis			cAMP level	
		30° C		42° C	Experiment 1	Experiment 2
		− cAMP	+ cAMP	− cAMP		
WZ11	*cya*$^+$ *alt*$^+$	100	100	100	197	231
WZ22	*cya*-855 alt$^+$	3.2	103	3.3	28	33
WZ25	*crp*-868 *alt*$^+$	2.7	2.6	3.0	201	213
WZ30	*cya*-855 *alt*-1	15	33	3.2	37	36
WZ57	*cya*$^+$ *alt*-1	47	48	46	—	—
Blank	—	—	—	—	16	24

(a) Cyclic AMP was assayed in cultures grown at 30° C from which perchloric acid extracts were prepared as described in Materials and Methods. After fractionation on PEI cellulose with LiCl, the amount of label in the spot corresponding to cyclic AMP was counted as described. Values given are the number of counts per minute per spot. The blank value was obtained by adding label to a perchloric acid extract of unlabelled WZ11.
(b) Each value for β-galactosidase represents the increase of enzyme activity synthesised per unit increase in OD (Klett units, red filter) of the assayed culture. These values are expressed as a percentage of the wild type activity in that growth condition.
(c) Cultures of the wild type grown with cAMP (10^{-3} M) show almost twice the β-galactosidase activity found in an equivalent culture without cAMP. This is due to relief of catabolite repression in these glucose-grown cultures (Perlman *et al.*, 1969; deCrombrugghe and Pastan, 1969).
(d) Note. We find β-galactosidase synthesis by WZ30 to be unchanged after it has been transduced to rel$^+$.

crp-868 is cotransducible with *strA* (Perlman, Chen, de Crombrugghe, Emmer, Gottesman, Varmus and Pastan, 1970).

Revertants of WZ22 and WZ25 occur at a frequency of 10^{-6} to 10^{-7}. They are readily selected on minimal agar containing arabinose as sole carbon source, since utilisation of this sugar requires the CRP-cAMP system (de Crombrugghe, Perlman, Varmus and Pastan, 1969). Some Ara$^+$ revertants continue to express other CS operons at a reduced level. They could have a mutation in the *ara* promoter circumventing its requirement for the CRP-cAMP system. The remainder regain function of the other CS operons. These pleiotropic revertants have therefore acquired a mutation which either restores or compensates for the CRP-cAMP system defective in these parent strains.

It is a remarkable fact that about 5% of the pleiotropic revertants are unable to grow at 42° C, either on minimal-glucose, or nutrient medium. We call these *alt* mutants (alternative to CRP-cAMP). Their properties form the basis of this investigation.

The Genetic Identity of Alt Mutants. We have studied 75 independent *alt* mutants isolated as revertants of *cya*-855 and *crp*-868 strains. Mating experiments show (Table 3) that the *alt* mutation in every case is located in the small sector of the *E. coli* chromosome between *metC* (59 minutes) and *spc* (64 minutes).

The *alt* gene is transferred early by Hfr ED1009 but not by Hfr ED1002. It is carried by the chromosomal fragment of KLF2 (Table 3). The *alt*-1 mutation does not cotransduce with *argG* or *pnp* (data not shown), indicating that the *alt*

Table 3. The mapping of *crp* and *alt* mutations by conjugation

Recipient genotype	No. of isolates tested	Donors yielding wild-type recombinants		
		KLF2	ED1002	ED1009
metB ***crp-868*** (WZ25)	1	−	+	+
metB cya-855 ***alt-1*** (WZ30)	1	+	−	+
metB crp-868 ***alt:*** *metB cya*-855 ***alt***	74	+	−	+

In each cross the donor marker tested for transfer is indicated in bold type.
(a) *The Hfr crosses.* From each cross we selected *met*B+ recombinants, which were then tested for the *crp*+ or *alt*+ character. + = more than 70% of met+ inheriting the donor marker. − = less than 1%.
(b) *The KLF2 crosses.* The *alt*+ derivatives were selected directly at 42° on minimal plates containing methionine and glucose. The KLF2 donor yields 10^4 *alt*+ derivatives per ml of mating mixture (parent input $\Omega 2 \times 10^8$ cells per ml). In a control cross the same donor yields 5×10^4 *arg*G+ derivatives per ml. The *alt*+ derivatives transfer KLF2 and segregate *alt*− at a low frequency (10^{-3}).

gene is very close to either *metC* or *spc*. In the case of one of the mutants, *alt*-1, we have shown that a single lesion is responsible for temperature sensitivity and the restoration of CS operon expression. Spontaneous temperature-resistant revertants were selected from WZ30 *cya*-855 *alt*-1. All of these (660) concomitantly became arabinose negative. If the two phenotypes were due to separate lesions, most, if not all our derivatives should have remained Ara+.

Do Alt Mutations Restore the CRP-cAMP System? The *alt*-1 mutation does not change either of the known factors (CRP and cAMP) required for normal transcription of CS operons. If the mutation changed the CRP protein enabling it to act without cAMP, *alt*-1 should map in the *crp* gene. The *crp* gene is located close to *strA* (Perlman *et al.*, 1970) and is transferred early by Hfr ED1002 (Table 3). As we have seen Hfr ED1002 does not transfer *alt* early. In addition we can show that *alt*-1 does not open a new route for cAMP synthesis. The strain WZ30 *cya*-855 *alt*-1 contains no more detectable cAMP than WZ22, its *alt*+ parent (Table 2). Moreover *alt*-1, isolated as a suppressor of *cya*-855, will also suppress the *crp*-868 mutation. Suppression of both *cya* and *crp* mutations would not be expected if *alt*-1 affected cAMP synthesis.

Since we find that *alt*-1 has no direct effect on CRP or cAMP, we conclude that it provides an alternative mechanism enhancing the expression of CS operons.

Does Alt-1 Affect Messenger Synthesis from CS Operons? Strains defective for the CRP-cAMP system synthesise a low amount of β-galactosidase (Table 2). The effect of the *alt*-1 mutation is to increase 3–5 fold the β-galactosidase synthesised by such strains (Table 2). The increment disappears when a *cya*-855 *alt*-1 culture is raised to 42° C (Table 2 and Fig. 2). Thus, the process permitting excess enzyme synthesis is temperature sensitive. We can ascribe the excess synthesis either to more efficient translation of the residual *lac* messenger in *cya*− strains or to an increase in the amount of *lac* messenger.

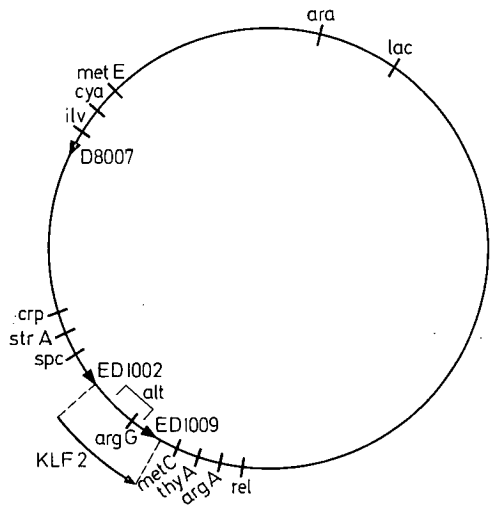


Fig. 1. A genetic map of *E. coli* showing the markers, Hfrs and F-prime factors used in this study. The two Hfr strains were formed by integration of F_{ts} lac into the bacterial chromosome (Masters and Broda, 1971). The F′ factor, KLF2, is also called F102. Its chromosomal fragment does not extend as far as *str*-A (Low, 1968; Goman, unpublished). This confirms the separate identities of the *alt* and *crp* genes, since *crp* maps beyond *str*-A (Goman, unpublished) and KLF2 carries *alt*

Results reported below show that the synthesis of certain messengers is temperature sensitive in *alt*-1 strains. Thus it is reasonable to infer that *alt*-1 stimulates β-galactosidase synthesis by increasing the synthesis of *lac* messenger.

A convenient technique for detecting the total messenger RNA synthesised by a bacterial culture has recently been devised (Pato and von Meyenberg, 1970). The culture is challenged with rifampicin, which rapidly prevents further initiation of RNA chains. Chains initiated before the drug is added complete their synthesis. They are labelled with ^{3}H-uridine, added at the same time as the drug. A fraction of this label is contained in unstable molecules, presumably messenger, which can be seen to disappear during further incubation of the culture. The remaining label is in stable RNA (Fig. 3).

At 42° C, a broth culture of WZ30 *cya*-855, *alt*-1, stops making detectable unstable RNA, while stable RNA continues to be made (Fig. 3). Such a culture ceases to grow (OD) within one generation after the shift. These observations suggest that a step necessary for messenger, but not stable RNA synthesis is rendered temperature sensitive by *alt*-1. We can be sure that this temperature sensitivity is caused by *alt*-1 since WZ22, the *cya*-855 *alt*$^+$ parent of WZ30 can make unstable RNA in broth at 42° C (Fig. 3 A).

Not all messengers require the *alt*-1 product for their synthesis. They can be detected in a minimal-grown culture of WZ30. In such a culture synthesis of detectable unstable RNA remains virtually unchanged for more than an hour (1.2 generations) after the shift to 42° C (Fig. 3 D), and growth stops only after 4 generations. This difference can be explained if we recall that growth in glucose-minimal medium demands derepression of all biosynthetic pathways; it seems

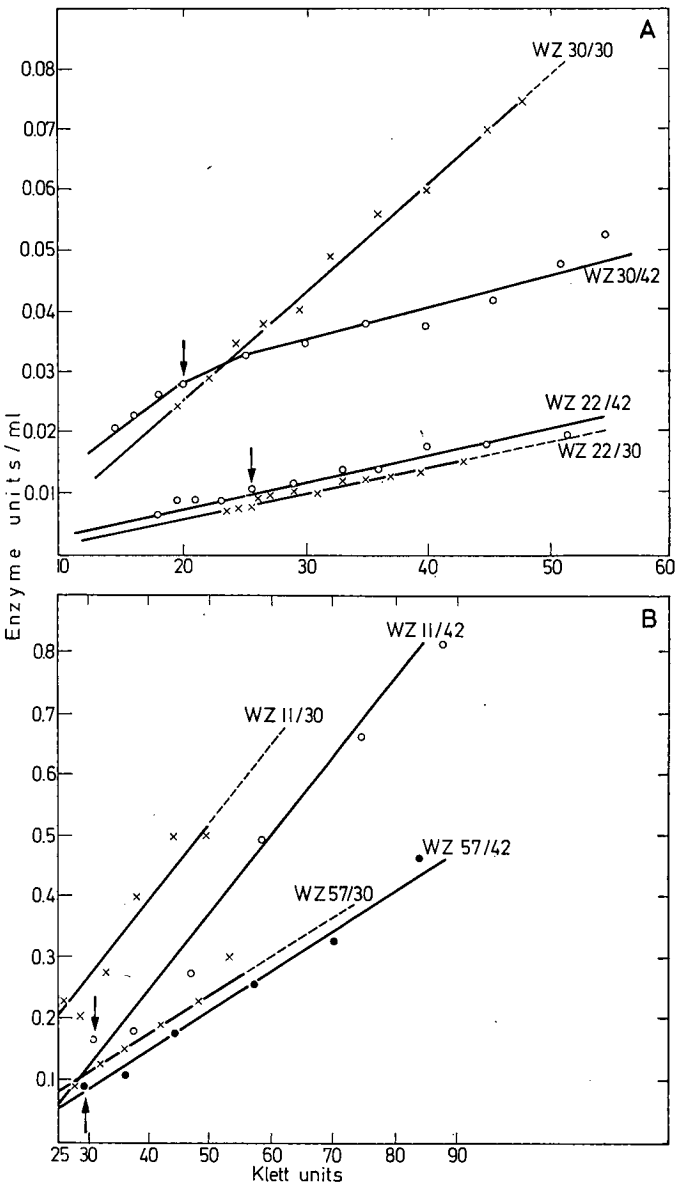


Fig. 2A and B. β-galactosidase synthesis at 30° C and 42° C in wild type, *cya*-855, and *alt*-1 strains. A, Strains WZ22 *cya*-855 and WZ30 *cya*-855 *alt*-1. B, Strains WZ11 *cya*$^+$ *alt*$^+$ and WZ57 *cya*$^+$ *alt*-1. Cultures were pregrown in supplemented MS-glucose $+10^{-3}$ M IPTG at 30° C. They were then diluted in the same medium at 30° C, allowed to resume exponential growth to the Klett values indicated, and then sampled and assayed as described in Materials and Methods. Cultures were shifted to 42° C at the optical density indicated by ↓. 30° C (×); 42° C (○, ●)

that synthesis of messenger from biosynthetic operons does not require the *alt* product.

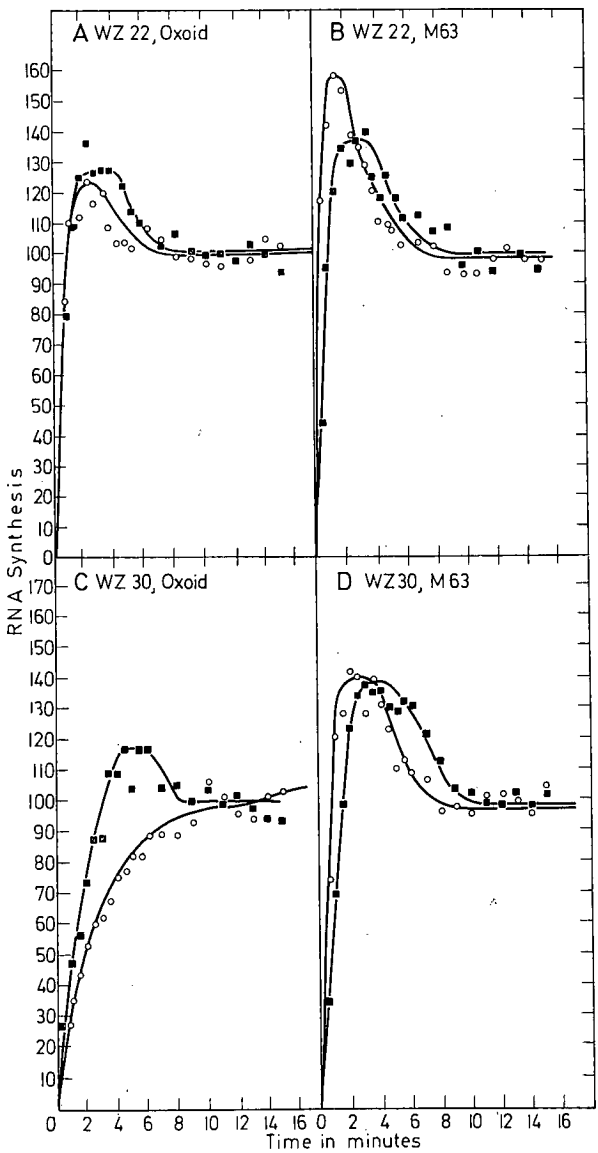


Fig. 3 A—D. Measurement of total RNA synthesis by WZ22 and WZ30. Cultures were maintained in the exponential phase at 30° C, and incubated at 42° C for 1–1.6 generations. An aliquot (2.5 ml) was taken from each culture and exposed to rifampicin (200 μg/ml) and ^{3}H-5-Uridine at 0 minutes. Samples (0.1 ml) were taken at the times indicated, prepared as described in Materials and Methods, and counted. Values for RNA synthesised are counted as a percent of the average plateau value for the particular experiment. 30° C control culture (▪); 42° C culture (○)

Our pulse-label experiments have enabled us to detect a class of messengers whose synthesis requires the *alt*-1 product. We therefore conclude that in selecting strains regaining CS operon activity, we have isolated mutants in which tran-

scription of CS operons is restored. This renewed transcription is determined by the *alt*-1 product.

The Mode of Action of the Alt Product. The evidence we have presented indicates that the *alt*-1 product is a factor which permits transcription of CS operons. In order to test whether the *alt* product normally participates in CS operon transcription we have examined the behaviour of a strain combining a normal CRP-cAMP system with the temperature sensitive *alt*-1 product. We have assayed synthesis of β-galactosidase in a culture of WZ57 *cya*$^+$ *crp*$^+$ *alt*-1 shifted from 30° to 42° C. We find that the temperature shift has no effect on the relative rate of β-galactosidase synthesis (Fig. 2 B). The simple interpretation of this result is that the *alt* product (in its heat-labile, *alt*-1 form in this strain) does not normally participate in CS operon transcription.

The Character of Alt-1 Mediated Transcription. Our mutant WZ22 *cya*-855, which contains little or no cAMP, expresses its CS operons much less efficiently than its *cya*$^+$ parent. This agrees with the conclusion (de Crombrugghe *et al.*, 1969; Zubay *et al.*, 1970) that maximum CS operon expression depends on the cAMP concentration in the bacterial cell. The residual enzyme synthesis in *cya*$^-$ strains is resistant to transient and catabolite repression, effects attributed in *cya*$^+$ strains to the reduction of the intracellular cAMP concentration. This resistance is shown in the following way.

The strain WZ47 is a glycerol specific revertant of strain WZ22. It can grow on glycerol and glucose-minimal media, but it cannot grow on other carbon sources such as lactose or arabinose. The mutation conferring this new property maps near the *glpK* locus (Silverstone, unpubl.) and is probably similar to the *lac* p^r_{UV5} mutation or the *glpK* mutation described by Berman and Lin (1971). Addition of glucose to a glycerol-growing culture of strain WZ47 does not affect the differential rate of β-galactosidase synthesis detectably (Fig. 4). On the other hand, addition of glucose to a glycerol-growing culture of strain WZ30 *cya*-855 *alt*-1, results in the immediate arrest of β-galactosidase synthesis known as transient repression (Magasanik, 1970). After one half a generation, β-galactosidase synthesis then resumes at half the differential rate in glycerol growing cells (catabolite repression). Therefore, the new system for transcription of CS operons appears to be subject to the glucose effects of catabolite and transient repression, although there is no detectable cAMP in the cells. We conclude that catabolite and transient repression can be mediated in *E. coli* by a substance or substances other than cAMP. This conclusion is reminiscent of the recent finding (Prival and Magasanik, 1971) that *Klebsiella aerogenes* has a system other than the CRP-cAMP system, which can promote initiation of transcription of some CS operons in response to changes in growth conditions.

Discussion

Our studies permit us to draw a number of firm conclusions about the *alt* function. The product is almost certainly a protein, since our *alt* mutants are temperature sensitive. It is a product which is vital to the cell, since, all tested *alt*-1 strains are unable to grow on any medium at 42°, while an *alt*-1/KLF2 *alt*$^+$ partial diploid can grow at this temperature. The *alt*$^+$ product mediates in the synthesis of certain RNA species and we can explain the temperature-sensitivity

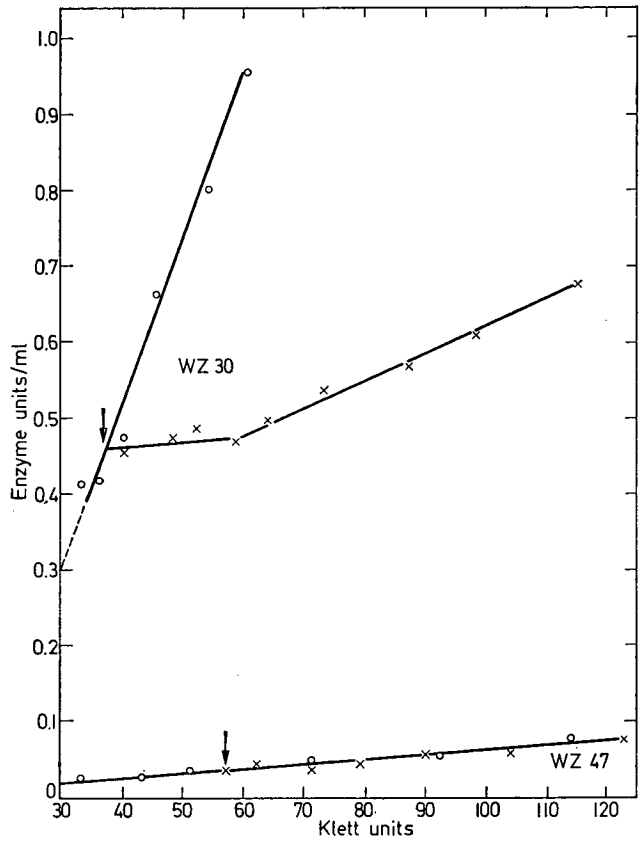


Fig. 4. Catabolite and transient repression of WZ30 and WZ47. Cultures were grown in MS-glycerol at 30° C and assayed as described in Fig. 2. Glucose (0.2%) was added at the optical density indicated (↓). Glycerol grown cultures (o); Cultures to which glucose is adedd (×)

of *alt*-1 strains if we assume that at least one of these RNA species is vital to the cell.

We can draw some general conclusions about the nature of the RNA species whose synthesis requires the *alt* function. Firstly, we can be sure that the *alt* function is used for the synthesis of some CRP-independent RNA species, because a broth-grown *cya*$^-$ *alt*$^+$ strain (WZ22) synthesises unstable RNA which cannot be found in WZ30 *cya*$^-$ *alt*-1 at 42° (compare Fig. 3 A and C). The transcription of CRP-independent species may be the exclusive function of the *alt*$^+$ product. In this case we must assume that *alt*-1 is a mutation which permits the *alt* protein to recognise CS promoters without abolishing its original specificity. The fact that *lac* expression ceases to be temperature-sensitive in *crp*$^+$ *cya*$^+$ *alt*-1 strain supports this conclusion. However, we would like to emphasise that this result does not exclude the possibility that *alt*$^+$ does normally participate in *lac* transcription, that the *alt*-1 mutation enables it to promote *lac* expression in the absence of CRP or cAMP and that the *alt*-1 protein is stabilised on CS promoters by an intact CRP-cAMP system.

We can envisage several possible functions for the *alt* protein. The simplest hypothesis is that the *alt* protein is a factor which enables RNA polymerase to initiate transcription of certain genes, for example by causing the enzyme to bind to their promoters. Such a mechanism has been proposed for CRP action (Travers, Kamen and Schleif, 1970). There are, however, other possibilities. The *alt* protein could have an enzymatic function, modifying either the polymerase or promoters, to facilitate the initiation of transcription. Otherwise it could be a subunit of RNA polymerase. The subunit would determine, directly or indirectly, the promoter-affinity of the enzyme, and in the *alt*-1 mutant would show its temperature-sensitivity only on certain promoters.

Recent genetical and biochemical studies (Eron and Block, 1971; Arditti, Grodzicker and Beckwith, in press) indicate that the *lac* promoter contains a specific CRP target site, deleted in the p^- mutation L1. Experiments are in progress to determine whether *alt*-1 acts at this site, or at the RNA polymerase binding site characterised by the *lac* promoter mutation p^r_{UV5}.

Acknowledgements. We gratefully acknowledge gifts of rifampicin from Lepetit Pharmaceuticals Ltd. One of us (A.S.) is a Fellow of the Jane Coffin Child's Memorial Fund for Medical Research.

References

Arditti, R. R., Grodzicker, T., Beckwith, J. R.: Evidence for two sites in the *lac*$^+$ promoter region. J. Mol. Biol. In press.

Berman, M., Lin, E. C. C.: Glycerol specific revertants of a phosphoenol-pyruvate phosphotransferase mutant: suppression by the desensitization of glycerol kinase to feedback inhibition. J. Bact. **105**, 113–120 (1971).

Bremer, H., Yuan, D.: Uridine transport and incorporation into nucleic acids of *Escherichia coli*. Biochim. biophys. Acta (Amst.) **169**, 21–34 (1968).

Cashel, M., Gallant, J.: Two compounds implicated in the function of the RC gene of *Escherichia coli*. Nature (Lond.) **221**, 830–841 (1969).

Crombrugghe, B. de, Chen, B., Anderson, W., Nissley, P., Gottesman, M., Pastan, I., Perlman, R.: *Lac* DNA, RNA polymerase and cyclic AMP receptor protein cyclic AMP, *lac* repressor and inducer are the essential elements for controlled *lac* transcription. Nature (Lond.) New Biology **231**, 139–142 (1971).

Crombrugghe, B. de, Perlman, R. L., Varmus, H. E., Pastan, I.: Regulation of inducible enzyme synthesis in *Escherichia coli* by cyclic AMP. J. biol. Chem. **244**, 5828–5835 (1969).

Emmer, M., Crombrugghe, B. de, Pastan, I., Perlman, R.: Cyclic AMP receptor protein of *E. coli*: Its role in the synthesis of inducible enzymes. Proc. nat. Acad. Sci. (Wash.) **66**, 780–787 (1970).

Eron, L., Block, R.: Mechanism of initiation and repression of *in vitro* transcription of the *lac* operon of *Escherichia coli*. Proc. nat. Acad. Sci. (Wash.) **68**, 1828–1832 (1971).

Haan, P. G. de, Gross, J. D.: Transfer delay and chromosome withdrawal in conjugation in *E. coli* K12. Genet. Res. **3**, 251–272 (1962).

Lederberg, E., Lederberg, J.: Genetic studies of lysogenicity in *E. coli*. Genetics **38**, 51–64 (1953).

Low, B.: Formation of merodiploids in *rec*$^-$ recipient strains of *E. coli* K12. Proc. nat. Acad. Sci. (Wash.) **60**, 160–167 (1968).

Magasanik, B.: Glucose effects, inducer exclusion and repression. In: The Lac Operon, eds. Beckwith and Zipser. Cold Spring Harbor 1970.

Masters, M., Broda, P. M. A.: Evidence for the bidirectional replication of the *E. coli* chromosome. Nature (Lond.) **232**, 137–140 (1971).

Matney, T. S., Goldschmidt, E. P., Erwin, N. S.: The specificity of genomic sites affording registry with the fertility episomes in *E. coli*. Biochem. biophys. Res. Commun. **12**, 483–486 (1963).

Pardee, A. B., Jacob, F., Monod, J.: The genetic control and cytoplasmic expression of "inducibility" on the synthesis of β-galactosidase by *E. coli*. J. molec. Biol. **1**, 165–178 (1959).

Pato, M. L., Meyenberg, K. von: Residual RNA synthesis in *Escherichia coli* after inhibition of initiation of transcription by rifampicin. Cold Spr. Harb. Symp. quant. Biol. **35**, 497–504 (1970).

Perlman, R., Chen, B., Crombrugghe, B. de, Emmer, M., Gottesman, M., Varmus, H., Pastan, I.: The regulation of *lac* operon transcription by cyclic adenosine 3′,5′-monophosphate. Cold Spr. Harb. Symp. quant. Biol. **35**, 719–723 (1970).

Perlman, R. L., Crombrugghe, B. de, Pastan, I.: Cyclic AMP regulates transient and catabolite repression. Nature (Lond.) **223**, 810–812 (1969).

Perlman, R. L., Pastan, I.: Regulation of β-galactosidase synthesis in *E. coli* by cyclic adenosine 3′,5′-monophosphate. J. biol. Chem. **243**, 5420–5427 (1968).

Prival, M. J., Magasanik, B.: Resistance repression of histidine and proline oxidase during nitrogen limited growth of *Klebsiella aerogenes*. J. biol. Chem. **246**, 6288–6296 (1971).

Randerath, K.: Thin layer chromatography. New York: Academic Press 1966.

Revel, H., Luria, S., Rotman, B.: Biosynthesis of β-galactosidase controlled by phage-carried genes. I. Induced β-D-galactosidase biosynthesis after transduction of gene Z^+ by a phage. Proc. nat. Acad. Sci. (Wash.) **47**, 1956–1967 (1961).

Riggs, A. D., Reiness, G., Zubay, G.: Purification and DNA-binding properties of the catabolite activator protein. Proc. nat. Acad. Sci. (Wash.) **68**, 1222–1225 (1971).

Scaife, J., Pekhov, A. P.: The deletion of chromosomal markers in association with F-prime formation in *E. coli* K12. Genet. Res. **5**, 495–498 (1964).

Schwartz, D., Beckwith, J. R.: Mutants missing a factor necessary for the expression of catabolite sensitive operons in *E. coli*. In: The Lac Operon, eds. D. Zipser and J. R. Beckwith. Cold Spring Harbor 1970.

Silverstone, A. E., Arditti, R. R., Magasanik, B.: Catabolite insensitive revertants of *lac* promoter mutants. Proc. nat. Acad. Sci. (Wash.) **66**, 773–779 (1970).

Silverstone, A. E., Magasanik, B., Reznikoff, W. S., Miller, J. H., Beckwith, J. R.: Catabolite sensitive sites of the *lac* operon. Nature (Lond.) **221**, 1012–1014 (1969).

Taylor, A. L.: Current linkage map of *Escherichia coli*. Bact. Rev. **34**, 155–175 (1970).

Travers, A. A., Kamen, R. I., Schleif, R. F.: Factor necessary for ribisomal RNA synthesis. Nature (Lond.) **228**, 748–751 (1970).

Tyler, B., Magasanik, B.: Molecular basis of transient repression of β-galactosidase in *Escherichia coli*. J. Bact. **97**, 550–556 (1969).

Ullman, A., Monod, J.: Cyclic AMP as an antagonist of catabolite repression in *E. coli*. FEBS Lett., **2**, 57–60 (1968).

Yokota, T., Gots, J. S.: Requirement for cAMP for flagella formation in *E. coli* and *S. typhimurium*. J. Bact. **103**, 513–516 (1970).

Zubay, G., Schwartz, D., Beckwith, J. R.: Mechanism of activation of catabolite-sensitive genes. A positive control system. Proc. nat. Acad. Sci. (Wash.) **66**, 104–110.

Communicated by E. Bautz

A. E. Silverstone
M. Goman
J. G. Scaife
MRC Molecular Genetics Unit
Department of Molecular Biology
University of Edinburgh
King's Building
Mayfield Road
Edinburgh EH9 3 JR
Scotland