THE CONTROL OF INITIATION OF DNA REPLICATION IN ESCHERICHIA COLI

by

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To Mum and Dad

with affection and gratitude

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CONTENTS

CHAPTER 1

1.I. INTRODUCTION

One of the most fundamental unsolved problems in biology is to understand how any organism regulates the major events of the cell cycle : replication and segregation of the genetic material, and cell division. Since the genetic organisation of prokaryotes is by definition more simple than that of eukaryotes, the former have been extensively studied as model systems. Study of the bacterial cell cycle is important in its own right since it may throw new light on how to fight bacteria as infectious agents, and the increasing recognition of the importance of plasmids in many practical ways has lent even more direct importance to the study of DNA replication in bacteria. Plasmids have been found to be responsible for the spread of drug resistance in bacterial populations, and perhaps are agents of evolution. Recent advances in the field of *in vitro* DNA technology have added commercial importance to plasmids so that much effort is being expended on developing means of amplifying plasmid DNA at will.

Although the genetic organisation of eukaryotes is more complex than that of prokaryotes, covalently closed circular DNA has been found in animal cells, and it has been shown that the transfer of extra-chromosomal DNA from bacteria to plants occurs naturally. The implications of this for the genetic manipulation of agricultural crops give added weight to the importance of a thorough understanding of the control of replication of various types of replicons and the interaction of those control processes.

1.II. CHROMOSOME REPLICATION IN BACTERIA

It is now quite clear, on both genetic (see Bachmann et al., 1976) and physical (Cairns, 1963a,b) evidence that the Escherichia coli chromosome is a circular DNA molecule with a molecular weight of about 2.5×10^9 daltons (see Cooper and Helmstetter, 1968). The bacterial chromosome replicates semi-conservatively (Meselson and Stahl, 1958) from a unique origin (Lark et al., 1963; Yoshikawa and Sueoka, 1963), although two self replicating fragments of DNA have been isolated from the E.coli chromosome (Yasuda and Hirota, 1977; Diaz and Pritchard, 1978). The normal physiological origin oriC is located at 82 minutes on the E.coli genetic map, (Bachmann et al., 1976) and gives rise to bidirectional replication at all growth rates (Bird et al., 1972; Prescott and Kuempel, 1972; Rodriguez and Davern, 1976). The oriC regions from both E.coli and Salmonella typhimurium have now been sequenced (Sugimoto et al., 1979; Meijer et al., 1979; Zyskind and Smith, 1980).

It now seems that the terminus of replication is a real structure rather than merely the point where two replication forks meet, since this region severely retards the passage of replication forks (Kuempel et al., 1977, 1978; Louarn et al., 1977) though this passage is not completely blocked (Kuempel et al., 1973). A similar retardation effect has been seen in the region of the terminus of plasmid R6K (Kolter and Helinski, 1978; Crosa et al., 1978; Figurski et al., 1978). The E.coli terminus of replication is thought to be between rac and man (Louarn et al., 1979) at a position approximately opposing the origin of replication on the E.coli genetic map.

The processes controlling replication in bacteria are not as well understood as the pattern of replication though considerable quantities of data have now been amassed concerning replication and the cell cycle. Pioneering work (Schaechter et al., 1958; Kjeldgaard et al., 1958) showed that while the relative proportions of protein, RNA and DNA are strictly regulated under steady state conditions, the proportion of DNA always took longer to adjust to a new nutritional equilibrium than that of the other constituents of the cell. Hanawalt et al. (1961) and Maaløe and Hanawalt (1961) found that susceptibility to thymineless death could be used as an assay for DNA synthesis. They found that the gradual halt in DNA synthesis seen upon amino acid starvation (Goldstein et al., 1959) or chloramphenicol treatment (Billen, 1959) was due to a reduction in the proportion of cells synthesising DNA. They also found that not all cells synthesise DNA at any given time in slowly growing cultures. This led to the proposition that initiation of a replication cycle occurs when a fixed DNA/mass ratio has been achieved and that DNA synthesis occurs at a constant rate within those cells making DNA (Maaløe, 1961). The problem of explaining how E.coli cells can grow at doubling times of less than 40 min when there is no gap in the replication cycle was solved by Sueoka and co-workers (Yoshikawa et al., 1964; Oishi et al., 1964) who found that the relative gene dosage (that is the frequency of one gene relative to an average gene) of markers near to the origin of replication is several times that of markers near to the terminus. Thus rounds of replication overlap temporally.

The powerful membrane elution technique (Helmstetter and Cummings, 1964) allowed the detection of discrete doublings in the rate of DNA synthesis, indicating a fixed period of DNA synthesis, and a fixed period before cell division (Clark and Maaløe, 1967). Similar experiments enabled Cooper and Helmstetter (1968) to describe the cell cycle in a formal manner. They defined the time taken to replicate a chromosome as C ($\sim$ 40 min) and the time between termination of a replication cycle and subsequent division as D ($\sim$ 20 min). The causal relationship between chromosome replication and cell division is not clear though it seems that initiation of the two events is linked (Meacock and Pritchard, 1975; Koppes et al., 1978). For a more exhaustive review of the cell cycle see Pritchard (1974). The length of the C period in slowly growing cultures is still disputed. Recent hybridisation studies have provided evidence supporting the belief that the length of the C period is invariant (Chandler et al., 1975), but such methods are by their nature insensitive at slow growth rates. Evidence from synchronised cultures suggests that at generation times longer than 60 min the C period lengthens, eventually approaching $\frac{1}{3}$ of the generation time at very slow growth rates (Helmstetter et al., 1968; Gudas and Pardee, 1974; Helmstetter and Pierucci, 1976; Kubitschek and Newman, 1978). The C time certainly may be varied by limitation of thymine (Pritchard and Zaritsky, 1970; Zaritsky and Pritchard, 1971; Bird et al., 1972) and by certain mutations (Lane and Denhardt, 1974).

Many models for the control of initiation have been produced, one of the first being that of Jacob and Brenner (1963; Jacob et al., 1963). They suggested that there was an operator of initiation, the replicator,

and that this operator was activated by an initiator protein. The structural gene for the initiator protein was in some way activated by the growth of a membrane site to which it was attached. This is claimed to be a model of positive control of replication, though if the initiator gene were negatively controlled this would be an interesting point of semantics. The model is useful in that it explains partition of chromosomes and plasmid incompatibility since each replicon is supposed to have its own membrane attachment site. There is considerable evidence that the origins, termini and replication forks of the Bacillus subtilis and E.coli chromosomes are associated with the cell membrane (Sueoka and Quinn, 1968; Snyder and Young, 1969; Yamaguchi and Yoshikawa, 1973, 1975, 1977; Sueoka and Hammers, 1974; Beeson and Sueoka, 1979; Fielding and Fox, 1970; Parker and Glaser, 1974; Nicolaidis and Holland, 1978; Hirota et al., 1980), and at least one plasmid has been found associated with the membrane of Bacillus subtilis (Winston and Sueoka, 1980a). It has been shown that these associations require active dna gene products in Bacillus subtilis (Winston and Sueoka, 1980b), and several specific proteins have been implicated in the attachment in E.coli (Kohiyama et al., 1977; Craine and Rupert, 1978; Wolf-Watz and Norqvist, 1979). Nonetheless it is not clear that such an attachment is connected with the control of initiation rather than having a more general role in replication, especially as the terminus region and replication fork of the chromosome seem to be membrane associated, and since Craine and Rupert (1979) have claimed that at least one form of origin-membrane complex is not required for initiation in E.coli. It has been suggested as part of a model for

plasmid incompatibility (T. Hashimoto-Gotoh, personal communication) that control of initiation may be effected by an inhibitor dilution mechanism (see below) whilst membrane attachment is necessary for replication and partition. The above findings and the reports that attachments persist throughout the cell cycle in E.coli (Craine and Rupert, 1978; Yaffe et al., 1979) are consistent with such an idea.

Following the demonstration by Donachie (1968) that there is a constant origin/mass ratio at initiation, thus confirming the prediction of Maaløe (1961; Maaløe and Hanawalt, 1961), a number of other models were produced as attempts to explain this tight correlation between cell mass and initiation. One of the first to be well described was that of Pritchard (1968; Pritchard et al., 1969). This proposed that replication is prevented by a stable inhibitor which is diluted by growth of the cell. At a critical concentration of inhibitor, initiation is allowed and a burst of fresh inhibitor is synthesised such as to double the cellular concentration. Again this model may explain the control of any replicon and the specificity of the inhibitor may determine plasmid incompatibility groups (see section 1.III).

A number of other models have been proposed, some involving the synthesis of an initiator, or replisome, which is synthesised constitutively (Helmstetter et al., 1968; Bleeken, 1971) or under auto regulation (Sompayrac and Maaløe, 1973). A model similar to the inhibitor dilution model of Pritchard (1968; Pritchard et al., 1969) was also proposed by Rosenberg et al. (1969). This model differed from the former chiefly in that it relied upon the active, constitutive synthesis of an anti-repressor, rather than dilution to remove the pulse of inhibitor made at initiation.

In view of the multitude and diversity of models, of which those mentioned here are not an exhaustive list, it may be useful to attempt to classify models into broad categories, according to their properties. Those models that rely upon constitutive synthesis of an initiator, or antirepressor may be classified as "unstable control" mechanisms. A reduction in the DNA concentration, perhaps due to thymine starvation, or due to statistical variations in the timing of initiation would lead to a reduction in gene concentration and thus in the rate of initiator synthesis, since constitutive gene output (defined as the rate of gene product synthesis) is proportional to gene concentration (Chandler, 1973; Chandler and Pritchard, 1975). This in turn would lead to a reduced rate of initiation upon restitution of thymine, and to a further fall in gene concentration. The negative control models, and this is taken to include any autoregulated control, predict self regulation of initiation. Late initiation in one round of replication will give early initiation, in terms of cell age, in the next. Such mechanisms thus give "self compensating control".

Another important distinguishing characteristic is the nature of the cellular parameter controlling initiation. We may define one group of models (which I will designate group A) that link the number of initiations in a given period to the amount of protein synthesised, and so to cell growth, during that same period. These models therefore depend upon <u>regulation of the rate of initiation</u> per replicon per unit cell growth. An example of this is the model of Helmstetter (1968) which involves constitutive synthesis of an initiator protein. Models involving autoregulation of initiator production are still group A as

they also regulate the *frequency* of initiation. The second class of model (which I will designate group B) rests upon regulation of the origin concentration, or number of origins per unit cell volume. The unstable inhibitor model of Pritchard et al. (1969) involves direct regulation of origin concentration, since according to this model each origin constantly produces a metabolically unstable inhibitor of initiation. Using this model we would predict that if it were possible to remove one origin from the cell by micro surgery, the cell would instantly recognise the loss due to a fall in inhibitor concentration and initiation of a remaining origin would result. The inhibitor dilution model (Pritchard, 1968; Pritchard et al., 1969) could not lead us to this prediction since it is based upon a mechanism of titrating the number of origins replicated over preceding generations rather than the number present at any instant. Under steady state growth conditions this fine distinction is irrelevant. The basic point to be made is that both of these class B models measure the number of origins replicated per generation and thus the origin concentration, rather than the rate of initiation or the number of origins initiated per generation. This is an important distinction to which I will return with reference to cointegrate replicons, in which systems initiation of one origin may lead to passive replication of another origin.

1.III. CONTROL OF PLASMID REPLICATION

Plasmids have been known for almost as long as E.coli genetics has been studied, and have now been found throughout the gram negative bacteria and in many of the gram positive bacteria. They confer many properties such as resistance to antibiotics, catabolic functions, bacteriocins, oncogenicity and biosynthetic functions, but from a replicative point of view, those found in the Enterobacteriaceae and studied in E.coli have been broadly classed into two types (see Rownd, 1978). Group I: the so-called "relaxed" plasmids, epitomised by ColE1 and cloDF13 are generally small (<10Md), and have a high copy number (10-20). Replication of these plasmids requires E.coli polymerase I and is probably unidirectional (Lovett et al., 1974) though it may be bidirectional in certain derivatives (Eichenlaub et al., 1979). This class of plasmids will continue to replicate for at least 14 hours in the absence of protein synthesis though the DNA thus formed may not be normal, and even E.coli chromosomal replication can continue in this way under certain circumstances (Kogoma and Lark, 1975). The "relaxed" plasmids are not normally self transmissible though they may be mobilised by conjugative plasmids.

The "stringent" plasmids (group II) are large (>25Md) conjugative plasmids with a low copy number (1-5 per E.coli origin). Replication in these plasmids is bidirectional (Eichenlaub et al., 1977; Rownd, 1978) and does not require host polymerase I. The "stringent" plasmids seem to have properties more like those of the E.coli chromosome and are therefore more likely to be of use in illuminating the problem of

control of chromosome replication. The most studied representatives of this group are the inc FI group as represented by the classical F plasmid and the inc FII group as represented by R100 (also called NR1 and R222), R1 and R6-5.

There are several plasmids that do not fit neatly into this classification. pSC101 is a small low copy number R plasmid which replicates undirectionally (see Cabello et al., 1976). R6K is a conjugative plasmid which has a high copy number (15-20), is of intermediate to large size (26Md) and has several origins of replication, each capable of giving rise to bidirectional replication. The normal origin of replication gives rise to bidirectional replication, replication of one arm being delayed relative to the other. There is a fixed terminus of replication which is not opposite the origin, (Crosa et al., 1976, 1978; Kolter and Helinski, 1978). Plasmid RK2 is of small size (38Md) and high copy number (5-7) for a "stringent" plasmid (Figurski et al., 1978). It is, therefore, clear that plasmids form a continuous spectrum and the designations "stringent" and "relaxed" are useful practical categories rather than of fundamental biological importance.

Pritchard (1978; Pritchard et al., 1969) has suggested that the control mechanisms acting on plasmid replication are similar to those acting on the bacterial chromosome and therefore they should show similar properties. Several studies on the copy number of the F plasmid using DNA-DNA hybridisation (Collins, 1971; Collins and Pritchard, 1973) or gene output (Pritchard et al., 1975) as a measure have shown that the F plasmid : chromosome origin ratio falls with increasing growth

rate and this has since been found to be true for the plasmids ColE1, R1, R6K, P1, pLT2 and several F-like R plasmids (Clewell and Helinski, 1972; Engberg and Nordström, 1975; Arai and Clowes, 1975; Prentki et al., 1977; Pritchard et al., 1979). Indeed the copy number (which I shall from here onwards use to mean copies per unit volume, or concentration) seems to change in a manner more like the terminus region of the E.coli chromosome than like the origin region. This is difficult to reconcile with the commonly accepted models for the control of chromosomal replication. Pritchard (1978) has suggested that this reflects not so much the nature of the control mechanism as the "tightness" of the control. A "sloppy" control would allow stochastic effects to play a large part in the control. The degree of rigidity of the control would depend upon the exact stoicheiometry of the control mechanism (see Sompayrac and Maaløe, 1973).

Nordström and co-workers have found evidence that they claim shows that plasmid replication is constant with time and independent of growth rate. They have found that in the case of plasmid R1, upon changing the growth rate of the culture, the rate of plasmid initiation does not change with time but instantaneously changes to a new rate with respect to protein synthesis (Gustafsson et al., 1978a). These workers also have found that when a temperature sensitive copy number mutant of R1 is shifted to a different temperature, the rate of replication adjusts immediately to a new rate (Gustafsson, 1977). These two findings together suggest that the rate of replication is controlled rather than the origin concentration, and that it is regulated independently of the growth rate. Control of R1 replication does not therefore seem to fit

any of the commonly accepted models for the control of chromosome replication except perhaps autoregulated production of initiator, (see section 1.II). These two treatments lead to a change in copy number and it has been suggested that they may be accommodated within the inhibitor dilution model in the same way as the change of copy number of F with growth rate (R.H. Pritchard personal communication). Thus these changes in rate of replication may reflect a changed probability of initiation at an uninhibited origin due to a change in concentration of another reactant involved in the initiation process.

One feature of plasmid replication that is in dispute is the timing and synchrony of replication with respect to the cell cycle. It seems clear that the so called "relaxed" plasmids replicate throughout the cell cycle (Rownd, 1969; Bazaral and Helinski, 1970) but there is dispute in the case of the "stringent" plasmids (see Pritchard, 1978). Nordström and co-workers claim that the timing of replication of R100 and Flac is random throughout the cell cycle on the basis of labelling studies of exponential cultures (Gustafsson and Nordström, 1975; Gustafsson et al., 1978b) whereas studies on synchronous cultures (Zeuthen and Pato, 1971; Cooper, 1972; Davis and Helmstetter, 1973; Finkelstein and Helmstetter, 1977) indicate that replication is timed at one point in the cell cycle. This may be important in the case of fast growing cultures where there may be several plasmid copies in the cell as it may alter predictions of the behaviour of cointegrates (see section 1.IV). It is not even certain that all of the chromosomal origins in the cell initiate synchronously. Most of the available

evidence suggests that they do (Helmstetter and Cooper, 1968; Kubitschek and Newman, 1978; Koppes et al., 1978) though the results of Lark et al. (1963) and Eberle (1968) are consistent with asynchronous initiation.

Certain of the models that invoke control of the initiation frequency predict that all origins should initiate synchronously (Jacob et al., 1963; Bleeken, 1971) but the majority of models do not. Copy number is determined by the stoicheiometry of the reaction (e.g. the exact size of the inhibitor pulse in the case of the inhibitor dilution model) but synchrony or lack of it is not predicted. Synchrony of replication can be accounted for by the inhibitor dilution model if it is assumed that inhibitor production is delayed after initiation (Pritchard et al., 1969). The important point here is that synchronous initiation will lead to a two-fold variation in copy number over the cell cycle whereas asynchronous replication will lead to a 0-100% variation depending upon the copy number.

The other important aspect of plasmid replication that is poorly understood is the phenomenon of incompatibility. It has been suggested that this is merely another reflection of the control mechanism and random segregation (Pritchard, 1978). This is supported by correlations between copy numbers and rates of segregation (Novick and Hoppenstadt, 1978; Ishii et al., 1978; Cullum and Broda, 1979). This is as expected if incompatibility is merely a manifestation of random segregation. Further support for this idea comes from the observation that conditional incompatibility (inc) mutants usually have an altered copy number, and also by studies on pSC134 (Cabello et al., 1976) which show that when

the copy number of plasmid pSC101 is artificially raised, its incompatibility (as measured by the rate of segregation) is also raised. Whilst incompatibility is clearly connected with replication it may well be an oversimplification to say that the one is merely a consequence of the other. There is evidence for a more complex mechanism. Three different complementation groups of inc mutants have been found in F (Manis and Kline, 1978; H.E.D. Lane, personal communication). It has also been shown by Meacock and Cohen (1979) that whilst all non-conditional inc mutants of pSC101 are defective in replication, replication defective mutants of pSC101 usually retain normal incompatibility functions.

A number of studies have been carried out which seem to disagree with the concept of a copy number titration mechanism in some plasmids. It has been found that under certain circumstances plasmids will reach a constant DNA concentration, rather than a constant copy number. ColE1 derivatives show this property in the presence of chloramphenicol. This is not surprising since chloramphenicol seems to inhibit the normal control mechanisms in E.coli (see section 4.II). Many copy number (cop) deletion mutants also show this property. This may simply indicate that the normal control mechanism has been inactivated and we are seeing a substrate limitation. In some cases it seems that a true secondary control mechanism is revealed in cop mutants (K. Nordström, personal communication). These problems still must be satisfactorily resolved but at present most of the available data seem consistent with the proposition that "stringent" plasmids at least share a similar type of control mechanism with the E.coli chromosome.

1.IV. COINTEGRATE REPLICONS

One approach that has thrown a great deal of light on the problem of control of initiation is the study of replicative properties of co-integrate replicons. It has often been stated authoritatively that in such cases one component of the cointegrate is inhibited or "switched off". This is not necessarily the prediction of most of the models for control of replication, and data on the subject are scant.

In the case of a cointegrate composed of two incompatible replicons, if incompatibility is an expression of the replication control mechanism, then it is always expected that only one replication system should be active at any one time, though the choice of origins may be random. In the case of compatible replicons, the predictions differ, depending upon the type of control model favoured. Most of the control models (class A as defined in section 1.II) involve the regulation of the frequency of initiation from an origin. This regulation would not be expected to be affected by the joining together of two replicons. Unless some special overriding control mechanism were proposed, it would be expected that each origin in the cointegrate would initiate at a frequency characteristic of the replicon from which that origin came. The second class of control models (class B as defined in section 1.II) demand that initiation from each origin will occur only with a frequency sufficient to maintain it at a minimum concentration with respect to bacterial cell volume. The inhibitor dilution model (Pritchard, 1968; Pritchard et al., 1969) as originally proposed states that replication through a gene at or near to the origin gives rise to

Table 1.1. Replication frequency and origin usage in pSC134

Strain description	Copy no (per genome equivalent)	% of replicating molecules initiating at ColE1 origin	% of replicating molecules initiating at pSC101 origin	Level of Tc resistance μgml^{-1}
polA$^+$(pSC101) [a]	5			25
polA$^+$(ColE1, pSC101) [a]	18+5			30
polA$^+$(pSC134) prediction type A model [b]	23	78	22	115
polA$^+$(pSC134) " " B " [b]	18	100	0	90
polA$^-$(pSC134) " both models [b]	5	0	100	25
polA$^+$(pSC134) [a]	16	> 97	< 3	80
polA$^-$(pSC134) [a]	6	< 7	> 93	25

(a) Experimental data taken from Cabello *et al*., 1976.

(b) Predicted figures calculated assuming normal copy nos. pSC101=5, ColE1=18 and pSC101, Tc resistance level of 25 μgml^{-1}, and that ColE1 replication is abolished in *polA*$^-$ strains.

a burst of inhibitor and as such it is a class B model in that it involves copy number titration rather than initiation frequency control. However if the model were modified to state that initiation per se gave rise to inhibitor synthesis (rather than a doubling in origin concentration) the model then becomes a class A model in that initiation frequency is titrated rather than origin concentration.

Class A models would predict that small cointegrates should have a copy number equal to the sum of those of the two individual replicons from which they are composed, since the total number of initiations will be the sum of those of the component parts. Class B models predict that the copy number would be equal to that of the component having the higher copy number in the autonomous state. A study distinguishing these two situations was undertaken by Cabello et al. (1976) who had previously joined up the high copy number plasmid ColE1 to the low copy number plasmid pSC101 to form plasmid pSC134 (Timmis et al., 1975). The results are summarised in Table 1.1. Briefly it may be seen that replication is from the ColE1 origin and the copy number is characteristic of ColE1 as predicted by a type B model. There are two remaining difficulties in this study. One is that due to the large difference in copy numbers a low level of replication from the pSC101 origin may have been masked, and the second is that the results are difficult to interpret since "relaxed" plasmids may have atypical replication properties.

Several artificial cointegrate plasmids have now been reported, these mainly being plasmid origins "cloned" into ColE1 derivatives. In most cases the replication properties have not been studied, but in three cases of plasmid cointegrates data have now come to light.

Figurski et al. (1978) have reported that a mini F-ColE1 cointegrate behaves qualitatively like pSC134 in that the ColE1 replication properties are dominant except in a polAts strain. When R6K is joined to ColE1, as expected the replication systems of both plasmids are active, and the copy number is high like that of both parents; the cointegrate may replicate in the presence of chloramphenicol like ColE1 and in a polAts strain at high temperature like R6K (Figurski et al., 1978; Crosa et al., 1978; Kolter and Helinski, 1978). The third case (Figurski et al., 1978) is anomalous and does not follow any predictions. Joining of the origin region of the low copy number plasmid RK2 (a P group plasmid) to the higher copy number plasmid ColE1, gives a low copy number cointegrate, whereas any of the models of replication control predict that the copy number should be equal to or greater than the copy number of ColE1. It seems that the selection procedure may have produced only plasmids in which the ColE1 replication machinery has been inactivated since the cointegrates are like RK2 in all their replication properties, being unable to replicate in the presence of chloramphenicol. The finding that even the insertion of non-replicating fragments of RK2 into ColE1 will prevent ColE1 from replicating in the presence of chloramphenicol suggests that there is some lethal genetic interaction between the two plasmids which is suppressed by mutations in the ColE1 replication region. There is some evidence in support of the idea that some lethal function is carried by the origin fragment of RK2 (Figurski and Helinski, 1979; Figurski et al., 1979).

It has also been reported that a number of naturally occurring plasmids such as F, NR1 and R6K have more than one origin of replication.

Normally in these plasmids only one origin is thought to function but up to three others may be induced to initiate when the host is deprived of thymine (Perlman and Rownd, 1976; Rownd, 1978; Crosa et al., 1976, 1978; Kolter and Helinski, 1978) or when small self replicating derivatives are isolated (Crosa et al., 1978; R. Skurray, personal communication; H. Lane, P. Bergquist and R. Gardner, personal communication). In some bacterial species, NR1(R100) will recombine to give several smaller plasmids (see Rownd et al., 1978). These findings indicate that the extra origins are not structurally inactivated since they are capable of functioning but are normally "switched off", probably because they are titrating their origin concentration.

Titration of origin concentration as a control mechanism is also supported by the finding that pSC134 has a stronger incompatibility effect on pSC101 than does pSC101 itself (Cabello et al., 1976). In this respect pSC134 is acting on pSC101 as Hfrs have been supposed to act on F plasmids.

Where cointegrates are made up of small replicons, such as most plasmids, the time taken to replicate the whole structure is small in relation to the minimum generation time of E.coli cultures. There is consequently a low probability of overlapping cycles of replication which would complicate predictions. In the case of Hfr strains of E.coli, the time taken for replication forks to traverse the chromosome may be several times the generation period. Thus if initiations should occur from the origins of both replicons the replication forks would meet at some point on the chromosome. This predicts that "copy number"

of the origin region should not be affected in most cases, but any initiation from the F origin of replication should lead to a change in the gradient of markers of the culture from that seen in an F^- strain. Only where the F plasmid is integrated into the E.coli chromosome at a point very close to the E.coli origin, oriC, do we expect no easily detectable change in the pattern of replication due to functioning of the F origin, but in this case we do expect the "copy number" or DNA/mass ratio of the culture to change since all of the replication forks will be travelling in the same direction around the chromosome.

A number of workers have investigated the pattern of replication in Hfr strains. One of the most recent and technically most convincing studies was by Chandler et al. (1976) who investigated both exponentially growing cultures and cultures starved of thymine for a short period. This treatment is known to prevent DNA replication without halting the growth of the culture, thereby leading to an accumulation of capacity to initiate (Pritchard and Lark, 1964). Replication should therefore occur from both the E.coli origin and the F plasmid origin upon release of the thymine restraint, unless there has been a structural inactivation of the F plasmid origin due to recombination into the chromosome to form the Hfr, or some later mutation. Chandler et al. (1976) showed that there was no such structural inactivation by demonstrating with a DNA:DNA hybridisation assay (see section 1.VII) that initiation did occur from both or either origin in the population. In contrast they claimed that in exponentially growing cultures they could detect no initiation from the F plasmid origin, though their data are consistent with a low level of initiation from the F plasmid.

Previous studies on the pattern of E.coli replication had involved less direct methods than the DNA hybridisation assay used above. Nagata (1962, 1963a,b) used the ratio of phage titres produced upon induction of a dilysogen to find the time of replication of the prophages in a culture synchronised by filtration. He claimed to find that replication proceded from the F origin of transfer in the opposite direction to the direction of transfer during mating, but could not find a consistent pattern in F^- strains. With our present knowledge of the position of the origin and of the bidirectional mode of replication in F^- strains, Nagata's data clearly show a disturbed pattern of replication in some Hfr strains. Berg and Caro (1967) used transducing lysates as a measure of relative gene dosage and could find no effect of the integration of the F plasmid upon the pattern of replication but later using density labelling of lysates they did find an effect with some Hfr strains (Caro and Berg, 1968). The data of Wolf et al. (1968a,b) obtained using a density labelling/transduction technique on Hfr DG163 showed evidence of initiation at the F plasmid origin as well as the E.coli origin. These experiments involving the use of bromouracil are, however, difficult to interpret in the light of the results of Chandler et al. (1976) since the experiments are not performed on exponentially growing cultures and because bromouracil is toxic to E.coli and may cause reinitiation of chromosome replication in the same way as thymine limitation (Wolf et al., 1968a).

Further information about the control of initiation in cointegrates comes from studies undertaken under conditions where one of the origins of replication is prevented from functioning. As expected the plasmid

pSC134, like pSC101, will replicate in a polAts strain of E.coli at the non-permissive temperature and has the copy number characteristic of one of its components : pSC101 (Cabello et al., 1976) see Table 1.1. The other component of pSC134, ColE1, which has a higher copy number cannot replicate in polAts strains at the high temperature. Thus as expected any "switch off" of the pSC101 origin is abolished when the higher copy number origin is inactivated causing the copy number to fall.

Similarly dnaAts strains of E.coli are defective in initiation of replication at the non-permissive temperature (see section 1.V) but temperature resistant "integratively suppressed" strains of E.coli and S.typhimurium may be isolated in which some plasmid has become integrated into the chromosome. This effect has been found to be mediated by F (Nandadasa, 1971; Nishimura et al., 1971) R100-1 (Nishimura et al., 1973) ColV2 (Nishimura et al., 1973) LT2 (Bagdasarian et al., 1975) many other F like plasmids (Moody and Runge, 1972) and I like plasmids (Datta and Barth, 1976), P1 and P7 (Chesney and Scott, 1978), P2 (Kuempel et al., 1977) and an increasing number of other plasmids. The original suggestion that replication in such strains may be initiated at the plasmid origin (Nishimura et al., 1971) has been confirmed (Bird et al., 1976; Chandler et al., 1977) for a strain integratively suppressed by R100-1 and for strains integratively suppressed by P2 (Kuempel et al., 1977). In the case of the former study replication was seen from the R100-1 origin even at the permissive temperature. This could be for several reasons. Since R100 has a higher copy number than F this may be expected. Such studies must be interpreted cautiously since further

mutations may be needed before a normal Hfr strain rendered *dnaA*$^-$ can be integratively suppressed (Nishimura *et al.*, 1971; Tresguerres *et al.*, 1975), probably because wild-type F plasmids cannot replicate at 42°C in *dnaA* strains (Tresguerres *et al.*, 1975). It is also known that integrative suppression by P1 and P7 is often associated with deletions and rearrangements of the prophage genome (Chesney *et al.*, 1979).

1.V. REPLICATION MUTANTS OF E.coli

Large numbers of E.coli mutants affected in the processes of DNA replication have now been isolated (for review see Wechsler, 1978). Of these mutants, some are affected in the process of initiation, and others are affected in ongoing replication or chain elongation. The latter are more numerous and the gene products of a number have now been isolated. Amongst these are the genes for DNA polymerase I (polA), DNA polymerase III (dnaE), DNA ligase (lig), elongation factors (dnaZ=dnaH) and pyrimidine metabolism (dnaS=dut).

Mutants affected in initiation classically show themselves as those in which a shift to non-permissive conditions causes the rate of DNA synthesis to tail off over a period of $\sim$ 40 minutes as rounds of replication complete. Several complementation groups have now been found, which include members that seem to be initiation mutants. One of the first to be isolated the dnaA mutants seem all to be affected in initiation (Hirota et al., 1968; Wechsler and Gross, 1971). Certain dnaC mutants are affected in chain elongation (Wechsler, 1975) whilst others are affected only in initiation (Carl, 1970; Wechsler and Gross, 1971). One single dnaB allele has been implicated in initiation (Zyskind and Smith, 1977), the other dnaB mutants, which are a numerous class, being defective only in elongation (Fangman, 1966; Fangman and Novick, 1968; Wechsler and Gross, 1971). Only one dnaP mutant is known, and may be defective in membrane synthesis (Wada and Yura, 1974; Wechsler, 1978). Of the two other classes of putative initiation mutants, dnaH is now known to be a dnaA thy double mutant (Derstine and Dumas,

1976) and dnaI may also be complex since it has been isolated only in one unusual genetic background (see Wechsler, 1978).

Most of these initiation mutants will reinitiate when returned to the permissive temperature but some dnaA mutants will not (Gross, 1972). These same strains seem to differ from other strains in various responses to chloramphenicol (Kogoma and Lark, 1975; Messer et al., 1975). The sensitivity of the reinitiation process to various antibiotics has been used to characterise the steps in initiation at which these mutants are blocked. Reinitiation of dnaC mutants is resistant to the inhibitors of protein and RNA synthesis, chloramphenicol and rifampicin, whereas dnaA reinitiation is sensitive to rifampicin and reinitiation of dnaB252 is sensitive to rifampicin and streptolydigin. This evidence has been claimed to show that the order in which the gene products act in the initiation process is dnaB, dnaA, dnaC (Zyskind and Smith, 1977; Zyskind et al., 1977). Similar conclusions were derived using dnaA heat sensitive, dnaC cold sensitive double mutant (Kung and Glaser, 1978). Despite some evidence to the contrary (Bayersmann et al., 1974; Zahn et al., 1977; Zahn and Messer, 1979) it now seems that dnaA mutations are recessive (Wechsler and Gross, 1971; Wehr et al., 1975; Derstine and Dumas, 1976; Gottfried and Wechsler, 1977; E. Orr, personal communication).

The dnaA phenotype may be suppressed by the integration of a number of plasmids into the chromosome (see section 1.IV) but the requirement of various plasmids for dnaA product is still not clear. Plasmid pSC101 seems to require $dnaA^+$ product (Hasunuma and Sekiguchi, 1977, 1979; Frey et al., 1979; Felton and Wright, 1979). F may require $dnaA^+$

(Tresguerres et al., 1975; Hiraga and Saitoh, 1975; Zeuten and Pato, 1971; Goebel, 1973) though these reports indicate that replication may continue at a decreasing rate for some time. There have also been conflicting reports concerning the requirements of the closely related plasmids ColE1 and cloDF13, (Goebel, 1973; Collins et al., 1975; Mayer et al., 1977; Veltkamp and Nijkamp, 1974; Frey et al., 1979). Replication of R100 and R1 seems to fall off only very slowly in the absence of active dnaA product (Goebel, 1973; Frey et al., 1979; Womble and Rownd, 1979a).

1.VI. THE EFFECT OF CHLORAMPHENICOL ON *dnaA* STRAINS

It has been shown that when temperature sensitive *dnaA* mutants are grown at temperatures above the permissive temperature, the DNA:mass ratio is progressively lowered (relative to that at the permissive temperature and to that of the wild-type) as the growth temperature is increased (Hansen and Rasmussen, 1977; Fralick, 1978a,b; Orr *et al.*, 1978; Pritchard *et al.*, 1978). It seems probable that this is due to a delay in initiation, since the rate of fork movement is not greatly reduced in such strains (Orr *et al.*, 1978; Tippe-Schindler *et al.*, 1979).

In certain *dnaA* strains, but not others, the gene product seems to be renaturable in that when cultures incubated at the non-permissive temperature are shifted to the fully permissive temperature, there is an immediate burst of DNA synthesis giving a rise in the DNA:mass ratio (Abe and Tomizawa, 1971; Blau and Mordoh, 1972; Hanna and Carl, 1975; Evans and Eberle, 1975; Messer *et al.*, 1975; Tippe-Schindler *et al.*, 1979). This burst of DNA synthesis seems to be proportional to the length of time at high temperature upto at least 1hr, (Hanna and Carl, 1975; Tippe-Schindler *et al.*, 1979). These strains seem to build up a capacity to initiate at the high temperature. Workers who were attempting to define which steps in initiation require protein and RNA synthesis found that rifampicin inhibits reinitiation at the permissive temperature, but chloramphenicol does not inhibit and may even stimulate DNA synthesis, though the effect of chloramphenicol may be highly concentration dependent (Blau and Mordoh, 1972; Evans and Eberle, 1975). This extra DNA synthesis induced by chloramphenicol may be due to extra

initiation since the final level of DNA synthesis after a shift back to 42°C is greater than in the absence of chloramphenicol (Blau and Mordoh, 1972).

A possibly related effect has been observed when strains are grown at a semi-permissive temperature, (a semi-permissive temperature being defined as that which allows steady state exponential growth, but with a reduced DNA:mass ratio). When chloramphenicol is added to such cultures in the absence of any temperature shift there is a burst of DNA synthesis, (Messer et al., 1975; Tippe-Schindler et al., 1979). This effect has also been attributed to reinitiation though the only evidence so far has been that the amount of residual synthesis is greater than can be attributed to termination of existing rounds of replication (Messer et al., 1975; Hansen and Rasmussen, 1977; Orr et al., 1978; Tippe-Schindler et al., 1979). Both the amount of DNA synthesised (Hansen and Rasmussen, 1977) and the rate of synthesis (Tippe-Schindler et al., 1979) seem to be proportional to the growth temperature, and the data of Hansen and Rasmussen (1977) show that in the absence of any temperature shifts, the amount of DNA synthesised by dnaA46 strains is just sufficient to make up the DNA:mass ratio to the wild-type level. Most workers agree that this represents a capacity to initiate, but the nature of this capacity is in dispute. Models of varying detail have been proposed to account for the cause and mechanism of this expression. One model says that decay of an unstable inhibitor of initiation is responsible for the reinitiation (Tippe-Schindler et al., 1979). A second model rests upon the proposition that an excess of inactive dnaA product is responsible for the effect (Hansen and Rasmussen, 1977)

but fails to explain why there is a stimulation in rate. In the third model it is proposed that a stimulation in the synthesis of an RNA species enables initiation to occur at a higher rate by a mass action effect (Orr et al., 1978).

The effect of other treatments on dnaA strains is in dispute, though some facts are clear. Amino acid starvation does not stimulate initiation, at least in dnaA46 strains, though there is such a stimulation in dnaA46 relA strains (Orr et al., 1978). No data have been published on the effect of amino acid starvation on other dnaA mutants though one group of workers claims that there is a stimulation of DNA synthesis in a dnaA5 strain (Tippe-Schindler et al., 1979) and another claims that there is none (Orr, personal communication). Any stimulation induced by rifampicin seems to be small, short lived and strain specific (Tippe-Schindler et al., 1979) and has not been observed in dnaA46 strains (Orr et al., 1978; Tippe-Schindler et al., 1979).

1.VII. METHODS USED TO INVESTIGATE CHROMOSOME REPLICATION

Several approaches have been used to study the pattern of chromosome replication. The direct visualisation of branched molecules is the simplest. Various ways of measuring the time of doubling of genes in synchronised, or age fractionated, or size fractionated cultures, have been useful in studying the sequence of replication of genes, and plasmids relative to each other and relative to the cell cycle. These methods have, unfortunately, usually involved some disturbance of the cell cycle during the synchronisation, or fractionation step. A means of studying the pattern of replication in undisturbed, exponentially growing bacterial cultures has been to measure marker gradients by assaying by some means the relative numbers of genes located at different points on the genetic map or the relative distribution of replication forks on the genetic map.

Virtually all of the studies on replication reported have followed one of these basic theoretical approaches but the techniques by which these have been carried out are legion.

Autoradiography and electron microscopy of DNA molecules are undoubtedly the most direct methods of investigating replication in a single cell. The frequently employed idea of measuring marker gradients suffers from the great weakness that it is only possible to measure the average marker gradient in a population as a whole. It therefore cannot readily distinguish between a population of cells, each having a bidirectionally replicating chromosome, and a population in which each

chromosome replicates unidirectionally but where the choice of direction is random. Since the majority of work undertaken has been marker gradient studies, this is one reason why the evidence from Cairns (1963a) autoradiographic study indicating that replication is unidirectional in E.coli was not readily refuted. It was further autoradiographic work which finally proved that replication is bidirectional within the individual chromosome as well as within the population (Prestcott and Kuempel, 1972; Rodriguez and Davern, 1976). Electron microscopy has been widely used to study the pattern of plasmid replication. Some recent examples are Lovett et al. (1974), Inselberg (1974), Tomizawa et al. (1974), Crosa et al. (1976, 1978), Cabello et al. (1976), Perlman and Rownd (1976), Eichenlaub et al. (1977) and Kolter and Helinski (1978). This method has not, however, been favoured for the study of bacterial chromosome replication.

Density labelling was used in some of the pioneering work on showing that replication is semiconservative (Meselson and Stahl, 1958) and combined with radioactive labelling of aligned cultures was used to demonstrate that there is a fixed but not necessarily unique origin of replication (Lark et al., 1963). Since then these methods have often been combined with some means of measuring relative gene dosage thus giving a more powerful technique. The finding that nitrosoguanidine causes mutations preferentially at the position of the replication fork has allowed studies of the pattern of replication to be performed in synchronous cultures of a number of bacterial genera (Cerdá-Olmedo and Hanawalt, 1968; Cerdá-Olmedo et al., 1968; Wolf et al., 1968b; Mishan'kin et al., 1973). A major disadvantage of these methods is that

cultures must be synchronised in some way which may impose an undesirable constraint upon the system under investigation. The use of toxic chemicals such as bromouracil and nitrosoguanidine is also undesirable.

The simple measurement of marker gradients in exponential cultures is more satisfactory than the above techniques; it is often the best method for looking at the kinetics of initiation and is complementary to autoradiography. The earliest studies of marker gradients were performed on Bacillus subtilis using transformation frequency as an assay of relative gene dosage. This was on the whole a satisfactory system and allowed Sueoka and coworkers (Yoshikawa and Sueoka, 1963; Yoshikawa et al., 1964; Oishi et al., 1964) to confirm that there is a fixed origin in exponentially growing cultures, and to demonstrate that in fast growing cultures, reinitiation may occur before the previous set of replication forks have terminated, leading to steep marker gradients. Unfortunately this method could not be applied to E.coli because of the technical difficulty of transformation, therefore a number of less direct methods were employed. The method of Nagata (1963a,b) relied upon synthesising a series of dilysogens and using the burst size as a measure of the relative gene dosages of the prophages. This method had some success but could never be entirely convincing because the induction of prophages must be expected to affect chromosome replication, and since the burst size must depend upon the physiological state of the cell.

Transducing lysates were used by several groups to measure relative gene dosages in E.coli as an analogous system to the transformation system in B.subtilis (Berg and Caro, 1967; Caro and Berg, 1968; Wolf et

al., 1968a,b; Masters and Broda, 1971). In most cases the method was combined with density labelling, in which case termini were preferentially labelled during amino acid starvation or origins preferentially labelled upon adding back amino acids and the heavy phage fraction separated by density gradient centrifugation. In cases where density labelling was not used, lysates from growing cultures were compared with lysates from aligned cultures to allow for the fact that all genes do not have similar recombination efficiencies. This type of control was also used in later hybridisation methods.

The output of constitutive or fully induced genes was also used to measure marker gradients or the time of doubling of genes in synchronous cultures with considerable success (Helmstetter, 1968; Wolf et al., 1968; Chandler, 1973; Chandler and Pritchard, 1975).

Despite the considerable quantity of work expended upon the above methods, by the early 1970's even the pattern of replication in E.coli had not been fully elucidated. The use of DNA-DNA hybridisation to measure the number of copies of any gene in a culture was a great break through because of its directness and hybridisation has been widely employed since its first use by Bird et al., (1972). They synthesised a number of isogenic mu: λ dilysogens having the mu prophage integrated at different points in the chromosome and used phage DNA as a hybridisation probe against DNA isolated from exponentially growing, or lined up cultures of the dilysogens. This method was finally successful in locating the origin. A neat variation of this method was later developed (Louarn et al., 1974). Cultures in which a synchronous cycle of replication had been induced, were pulse labelled at various times.

DNA from these cultures was then hybridised against filters loaded with an excess of unlabelled 'phage DNA to detect the time in the replication cycle at which the prophage DNA became labelled with optimal efficiency : this would be the time at which the prophage genome was replicated. These two variants together or individually have been used to locate the origin of replication (Bird et al., 1972; Louarn et al., 1974), to investigate whether the replication time (C) may be changed by thymine limitation (Bird et al., 1972) as deduced by Pritchard and Zaritsky (1970; Zaritsky and Pritchard, 1971), by rep mutations (Lane and Denhardt, 1975), or by slow growth rate (Chandler et al., 1975). They have also been used to investigate the pattern and frequency of initiation in cold sensitive dnaA$^+$ revertants (Kellenberger-Gujer et al., 1978) and in both normal (Chandler et al., 1976) and integratively suppressed (Bird et al., 1976) Hfr strains.

Kuempel et al. (1977, 1978) have recently used transducing 'phage, and F' plasmids as hybridisation probes to locate the replication terminus. Perhaps the most elegant variation on the hybridisation theme has also been used for this purpose (Louarn et al., 1977, 1979; Fayet and Louarn, 1978). This depends upon the property of certain 'phage DNA, that it may be separated into complementary strands. Knowing the orientation of certain prophages in the host DNA they were able to see which strand was complementary to Okazaki fragments and thus the direction of travel of the replication fork (Louarn and Bird, 1974).

1.VIII. CHOICE OF METHODS

It was the intention at the outset of this project to investigate the control of replication by studying the effect of one or a number of mutations, growth conditions or chromosomal rearrangements on the pattern of chromosomal replication. The most promising means of doing this seemed to be by DNA-DNA hybridisation. The only hybridisation methods that had then been applied were those of Bird et al. (1972) and Louarn et al. (1974). These methods are similar in that they use 'phage and prophage as the two homologous species. This necessitates the laborious construction of a series of isogenic dilysogens having prophage mu at different points in the chromosome, and the process of strain construction must be repeated every time that a different strain is to be investigated. Besides the labour problem, there is also the danger that by genetic manipulation of strains, the replication properties may be unwittingly altered.

The first aim of this project was therefore to develop a more versatile system that obviated the need to construct derivative strains. Recent advances in in vitro genetic manipulation suggested a means of doing this. If fragments of the E.coli chromosome could be used as hybridisation probes, large numbers of strains could be compared using the same probes. The chromosomal fragments would need to be covalently joined to a suitable plasmid or 'phage cloning vector, but this genetic manipulation need only be performed once, and would not affect the characteristics of the strains under investigation.

Several vector systems presented themselves. A number of groups were known to have "cloned" E.coli chromosomal or other fragments using ColE1 or its derivatives (Hershfield et al., 1974; Clarke and Carbon, 1975; Collins et al., 1976a; Collins et al., 1976b; So et al., 1975; Hershfield et al., 1976), pSC101 (Cohen et al., 1973) or lambda (Thomas et al., 1974; Borck et al., 1976), and λ dv was under development as a vector (Mukai et al., 1976). No complete collections representing the whole genome of E.coli had been produced, other than that of Clarke and Carbon (1974) which was made by the linking of randomly sheared DNA to ColE1 by polydA-polydT tailing. The use of restriction enzymes to cleave the DNA seemed more promising for the purpose of this study, since the chromosomal fragments could, should it prove necessary, be easily separated from the vector DNA. Since the growth and purification of DNA from different systems tested (Collins et al., 1976b; Borck et al., 1976) had its own idiosyncrasies, it was decided to "clone" a bank of E.coli genes using the amp^R derivative of ColE1, pSF2124 (So et al., 1975), as vector and to screen this bank for markers usefully positioned on the genetic map. It was hoped that this would provide a collection of probes, similar in source, structure and therefore in hybridisation properties.

Another problem with existing methods had been to obtain probe DNA of a sufficiently high specific activity (Pritchard and Chandler, personal communication). The "nick translation" system of Macgregor and Mizuno (1976) was therefore adapted to allow the efficient labelling of up to 200 μg of DNA in one reaction tube, to relatively high specific activities.

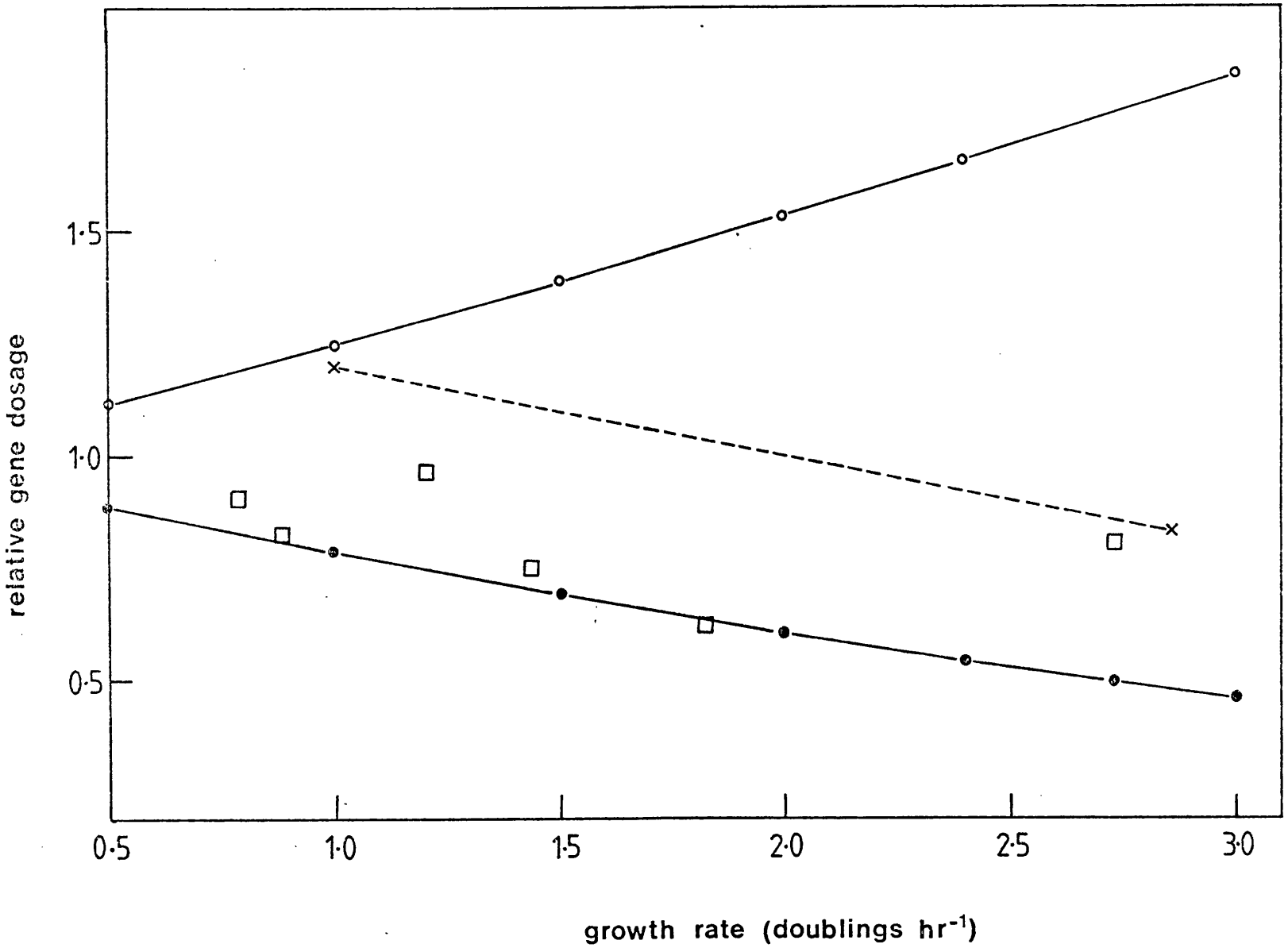
Armed with this new system it was hoped that the following problems could be tackled.

Figure 1.1. The relative gene dosage of autonomous F

The relative gene dosage of the F plasmid in the autonomous state at various growth rates is shown. The data are redrawn from Collins and Pritchard (1973) (×) or calculated from values of $^{F}/_{oriC}$ given by Pritchard et al. (1975) (□). Values for the relative gene dosage of the E.coli origin (○) and terminus (●) regions were calculated from the equation derived by Collins and Pritchard (1971):

$$\frac{F}{G} = \frac{C \ln 2.2^{C(1-x)/\tau}}{(2^{C/} - 1)}$$

where $\frac{F}{G}$ is the mean number of copies of a gene F per resting genome equivalent. C is the replication times of the chromosome and τ is the generation time. x is the fraction of the chromosome replication time C after initiation at which the gene F replicates. x is taken as being effectively the fraction of the distance from the origin to the terminus at which the gene maps (Bachmann and Low, 1980) and C is assumed to be 40 min.


relative gene dosage
1·5
1·0
0·5
0·5
1·0
1·5
2·0
2·5
3·0
growth rate (doublings hr⁻¹)

available data for the relative gene dosage of F within an F^+ cell with the relative gene dosage of E.coli origins and termini at various growth rates. Most of the studies undertaken on Hfr replication in exponentially growing populations have looked at Hfrs growing relatively quickly and those in which F is integrated into that half of the chromosome nearer to the E.coli origin of replication. The data of Collins (1971; Collins and Pritchard, 1973) suggest that the F origin is expected to be inactive in such cases. The converse has never been tested.

Due to the difficulty of measuring the shallow gradients of markers found in very slowly growing cultures, it was decided that replication should be investigated under slow growth conditions in an Hfr strain which has the point of insertion of F distant from the E.coli origin. Unfortunately there are no stable, and well characterised Hfrs having F inserted near to the E.coli terminus except for those made using F plasmids having temperature sensitive replication mutations (a fact which may possibly be of significance in itself). Hfr KL99, which is known to have given rise to F' plasmids (Low, 1972) was therefore chosen for study. DNA was isolated from KL99 and the closely related F^- strain KL1 growing at the same growth rate. The counts hybridising to each sample of DNA using any of a variety of E.coli:RSF2124 recombinant probes were expressed in relation to those hybridising using the RSF2124: $argE^+$ probe. The ratios found in the two growing strains were then compared. As a control, DNA was isolated from cultures which had been treated with rifampicin in order to block initiation and produce an identical gene dosage for all parts of the chromosome.

As a further control to see whether any "switch on" effect was

dependent upon the position of the integrated F plasmid in the chromosome it would be ideal to repeat the above experiment using an Hfr having F inserted near to the host origin. The problem is that no change in the pattern of replication would be expected in such a case. However, if the F plasmid were integrated very close to the E.coli origin the situation would become like that of a small plasmid cointegrate, where the two origins are close together in terms of replication time, thus a very significant rise in the copy number (or DNA concentration) would be expected in the case of a type A model, and none in the case of a type B. The DNA concentration was therefore measured in such an Hfr strain AB313 and in its isogenic F^- counterpart.

1.X. PROPOSED WORK 2 - THE EFFECT OF CHLORAMPHENICOL ON *dnaA* STRAINS

It has been largely accepted that the DNA synthesis induced by chloramphenicol treatment of *dnaA* strains growing at semi-permissive temperatures is due to initiation at the *E.coli* origin of replication. Evidence that it is initiation is indirect and depends upon the level of residual synthesis. Since the assay for initiation is so indirect it has not been possible to study the kinetics of initiation in any meaningful way, especially since it has not been shown that the C time is not altered under conditions of chloramphenicol treatment, and indeed there is some evidence that it might be affected (Rodriguez and Davern, 1976). In view of the findings that *dnaA* mutations may be integratively suppressed by a wide variety of replicons (see Chapter 1.IV), that several replicons may initiate in the presence of chloramphenicol under certain circumstances (Rosenberg *et al.*, 1969; Clewell, 1972; Kogoma and Lark, 1975; Womble and Rownd, 1979b), and that the *E.coli* chromosome contains at least one cryptic prophage which retains a functional origin (Diaz and Pritchard, 1978; Diaz *et al.*, 1979), it has also been open to question whether chloramphenicol induced initiation does occur from the normal *E.coli* origin *oriC*.

The method of investigating the pattern of replication developed as a part of this study offered a very appropriate means of settling these important questions. A selection of suitable hybridisation probes were therefore used to study the change in marker ratios at various times after the addition of chloramphenicol to a *dnaA46* strain, and a wild-type control growing at a semi-permissive temperature. In support

of these experiments the change in DNA content of the culture was also measured more directly than before by chemical estimation, and the rate of synthesis of DNA and RNA followed by pulse labelling.

CHAPTER 2

2.I. STRAINS AND MATERIALS

A. STRAINS

Strains and plasmids

The properties of all of the bacterial strains used in this study are summarised in Table 2.1. The properties of plasmids used for hybridisation are shown in Table 2.2. Fig. 2.1 shows the points of insertion of F in the Hfr strains used relative to the markers used in the hybridisation experiments.

B. MEDIA

Nutrient broth was from Oxoid. Nutrient agar was nutrient broth solidified with 1.25% agar.

L broth consisted of 1% tryptone, 0.5% yeast extract, 0.5% sodium chloride. Lagar was L broth solidified with 1.25% agar.

Tryptone broth was 1% tryptone, 0.5% sodium chloride.

Lambda tryptone agar was tryptone broth supplemented with 10mM magnesium sulphate and solidified with 1% agar. Lambda soft agar was similar but contained only 0.7% agar.

Table 2.1. Standard Strains

Strain	Genotype	Reference or origin	Source
A3	F⁻ thr-1 leuB6 thi-1 lacY1 supE44 dnaA46 λ^- λ^s	Tresguerres et al. (1975)	R.H. Pritchard
A3⁺	As A3 but dna⁺	Orr et al. (1978)	R.H. Pritchard
AB253	F⁻ thr-1 leuB6 thi-1 lacZ4 strA8 supE44	Bachmann (1972)	CGSC253[g]
AB313	As AB253 but Hfr (P013)	Bachmann (1972) Low (1972)	CGSC313[g]
AB1157	F⁻ thr-1 leuB6 thi-1 his-4 argE3 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 str-31 tsx-33 sup37 λ^- λ^s	Bachmann (1972)	R. Diaz
AB1623	F⁻ gltA ara gal lac thi		S. Harford
AB2463	As AB1157 but recA13	Bachmann (1972)	R. Diaz
AT713	F⁻ argA21 cysC43 lysA22 mtl-2 xyl-7 malA1 thi-1? strA108 supE44? λ^R λ^-		CGSC4529[g]
AT2092	F⁻ his-1 purF1 pheA2::Mu argH1 thi-1 lacY1 or 24 malA1 xyl-7 mtl-2 strA8 or -9 or -14 tonA2 or -14 tsx-23 or -25 supE44 λ^R λ^-?		CGSC3579[g]
BB20	As AB1157 but Δ(trpAB-tonB)[a]		W. Brammar

Table 2.1 Continued

Strain	Genotype	Reference or origin	Source
BB21	As AB2463 but Δ(trpAB-tonB)[a]		W. Brammar
C600	F⁻ thr-1 leuB6 thi-1 lacY1 tonA21 supE44 λ⁻ λ[s]	Bachmann (1972)	B.M. Wilkins
GMS343	F⁻ argE3 lacY1 galK2 mtl-1 man-4 strA700 tsx-29 supE44?[b]	Novel & Novel (1973)	CGSC5496[g]
GMS407	F⁻ argE3 lacY1 galK2 mtl-1 man-4 uidA1 tsx-29 supE44?[b]	Novel & Novel (1973)	CGSC5497[g]
JC1553	F⁻ metB1 leuB6 his-1 argG6 lacY1 or 24 malA1 xyl-7 mtl-2 gal-6 str-104 recA1 supE44? λ⁻ λ[R]	Bachmann (1972)	R. Diaz
JC5088	Hfr (P045) thr-300 ilv-318 recA56 spc-300 thi-1 relA1 λ⁻	Bachmann (1972)	R. Diaz
KL1	F⁻ thi-1 relA1 λ⁻	[c]	CGSC4395[g]
KL20	As KL1 but F⁺	Bachmann (1972)	CGSC4396[g]
KL99	As KL1 but Hfr (P042)	Bachmann (1972) Low (1972)	CGSC4242[g]
KL188	F⁻ thi-1 pyrD34 his-68 trp-45 thyA25 mtl-2 xyl-7 malA1 galK35 strA118 λ⁻ λ[R] sup[o]		CGSC4211[g]

Table 2.1 Continued

Strain	Genotype	Reference or origin	Source
LE404	C600 (pSC122, ColE1)		R. Diaz
LE405	C600 (pSC122)		R. Diaz
LE500	AB1157 (F'*lac*)	AB1157xRP273[e]	e
LE501	P162-8 (cI857Sam7)	Infection of P162-8 [d]	This study
LE515	As AT713 but *cys*$^+$ *recA56*	AT713xJC5088[d]	"
LE521	As AT2092 but *phe*$^+$ *recA56*	AT2092xJC5088[d]	"
LE529	As AB1623 but *thyA*	trimethoprim selection [d]	"
LE530	As GMS407 but *thyA*	trimethoprim selection [d]	"
LE536	As GMS407 but *recA56*	LE530xJC5088 [d]	"
LE543	As AB1623 but *recA56*	LE529xJC5088 [d]	"
P162-8	F$^-$ *thr-1 leuB6 thi-1 lacY1 deoC1 deoB1 thyA6 supE44? tonA21?* λ^-	Beacham *et al.* (1971).	R.H. Pritchard
W3110	F$^-$ λ^-	Bachmann (1972)	R. Diaz

Table 2.1 Continued — *In vitro* recombinant strains

Strain	Genotype	Reference or origin	Source
508	C600 Δ trpE(pVH5)	Hershfield *et al.* (1974)	R. Diaz
LE496	AB2463(pLG4)	h	"
LE497	AB2463(pLG5)	h	"
LE537	AT2092(pLG13)	Transformation with pLG13 from LE10095 [f]	This study
LE539	AB2463(pLG17)	Transformation with pLG17 from LE10214 [f]	"
LE541	LE536(pLG18)	Transformation with pLG18 from LE10268 [f]	"
LE562	LE521(pLG19)	Primary transformant (direct selection [f])	"
LE10065	LE500(pLG11)	Primary transformant (indirect selection [f])	"
LE10076	LE500(pLG20)	"	"
LE10095	LE500(pLG13)	"	"
LE10129	LE500(pLG14)	"	"
LE10214	LE500(pLG17)	"	"
LE10268	LE500(pLG18)	"	"
LE10378	LE500(pLG15)	"	"
LE10525	LE500(pLG16)	"	"
LE10684	LE500(pLG12)	"	"
AB2463 (pLG2)	AB2463(pLG2)	Diaz & Pritchard (1978)	R. Diaz

Table 2.1. Footnotes

a The deletions in BB20 and BB21 originated independently (W. Brammar personal communication).

b Originally described by the *E.coli* Genetic Stock Center as *xyl-5*? the isolates sent here were found to be *xyl*$^+$.

c KL1 was obtained by curing KL20 with acridine orange (B.J. Bachmann personal communication).

d See section on construction of strains.

e Isolated from a plate carrying transconjugants of AB1157 and RP273 (Tresguerres *et al.*, 1975). The plate was kindly provided by R. Diaz.

f See section 3.II.

g CGSC numbers indicate strains obtained from B.J. Bachmann. Curator of the *E.coli* Genetic Stock Center. The number is the strain number in that collection.

h Constructed by R. Diaz essentially by the method of Collins *et al.* (1976a) using RSF2124 and C600 DNA (R. Diaz personal communication).

Table 2.2. Fully characterised plasmids generated in this work and other probes used for hybridisation

Plasmid	Vector	Molecular weight of chromosomal fragment	Chromosomal marker carried	Map location of [b] chromosomal fragment	Reference
pLG2	[a]	8.5Md	*oriJ*$^+$	30	Diaz *et al.*, 1979
pLG4	RSF2124[d]	12.8Md [e]	*argE*$^+$	89	c
pLG5	"	7.5Md	*proA*$^+$	6	c
pLG17	"	7.5Md	*mtl*$^+$	80	
pLG18	"	6.5Md	*manA*$^+$	36	
pLG19	"	5.3Md	*purF*$^+$	49	
pVH5	ColE1	8.5Md [f]	*trp*$^+$	27	Hershfield *et al.*, 1974
λ318	λNM781	5.9Md	*oriC*$^+$	83	g

a self-replicating chromosomal fragment joined to non-replicative ampicillin resistance fragment.

b positions according to Bachmann and Low, 1980.

c constructed in a manner similar to that described herein for pLG19 (R. Diaz personal communication).

d see So et al., 1975.

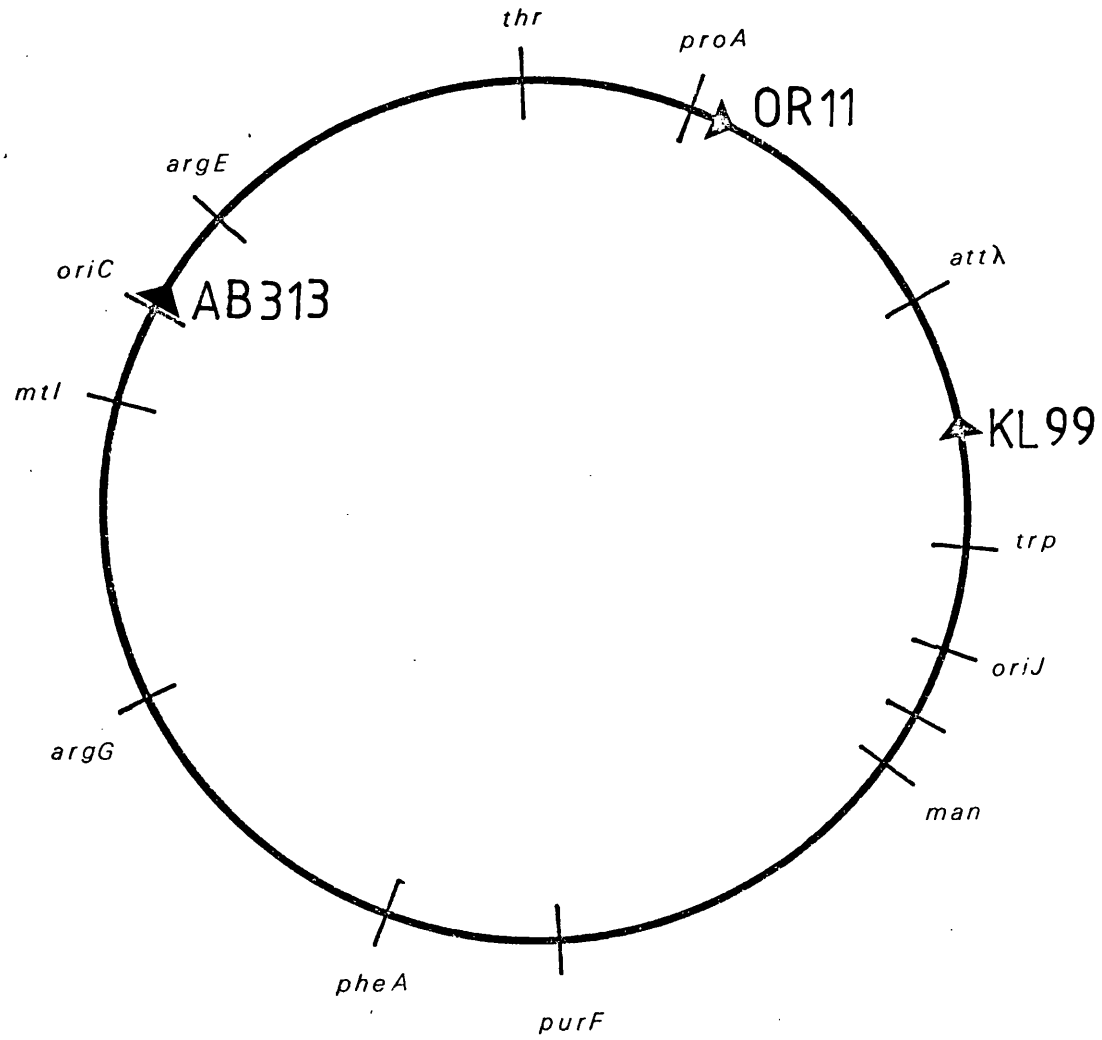
e believed to be similar to the chromosomal fragment of pNK1 (Kozlov et al., 1977).

f probably contains 'phage sequences.

g λ318 was constructed by the ligation of the chromosomal oriC fragment of pLG6 (Diaz and Pritchard, 1978) into λNM781 (Murray et al., 1977).

Figure 2.1. Genetic map of *E.coli* K12

The points of insertion of F in KL99 and AB313 are shown relative to chromosomal markers obtained for use as hybridisation probes.
Redrawn from Bachmann and Low, 1980.


thr
proA
OR11
argE
oriC
AB313
attλ
KL99
mtl
trp
oriJ
argG
man
pheA
purF

Tetrazolium agar was nutrient agar or Lagar supplemented with an appropriate sugar at 1.25% and tetrazolium salts at 0.015%.

MS2 agar had the following composition : tryptone (Difco) 1%, yeast extract (Oxoid) 1%, sodium chloride 0.8%, calcium chloride 2mM, glucose 1% and agar (Difco) 1.5%.

M9 medium (which was solidified with 1.5% agar where necessary) was made by diluting one volume of CM solution, ten volumes of M9x10, and one volume of a sterile 20% solution of glucose (or for selective purposes another appropriate sugar), into sterile distilled water or aqueous agar to make 100 volumes. Required amino acids and bases were also added where appropriate at a final concentration of 50 $\mu g ml^{-1}$ and thiamine at 10 $\mu g ml^{-1}$. Thymine was added at 50 $\mu g ml^{-1}$ unless otherwise indicated in the text. CM solution and M9x10 which were made up and autoclaved separately were:

M9x10 per litre, Na_2HPO_4 60g, KH_2PO_4 30g, NaCl 5g, NH_4Cl 10g.

CM solution per litre, $CaCl_2$ 1.1g, $MgSO_4.7.H_2O$ 24.1g.

M9 casamino acids medium consisted of M9 medium containing all necessary growth requirements and enriched with 0.5% casamino acids (Difco).

C. BUFFERS AND OTHER REAGENT SOLUTIONS

Lambda buffer was a sterilised solution of 6mM tris pH7.7, 10mM $MgSO_4$, 50 $\mu g ml^{-1}$ gelatin.

Gel denaturing buffer was 1.5M sodium chloride, 0.5M sodium hydroxide.

Gel neutralising buffer was 3M sodium chloride, 0.5M tris HCl pH7.0.

Tris-borate gel buffer contained per litre : tris base 10.8g, EDTA disodium salt 0.93g and boric acid 5.5g.

Tris-acetate gel buffer was 30mM tris-acetate, 1mM EDTA pH7.7.

SSC (standard saline citrate) is 0.15M sodium chloride, 0.015M trisodium citrate (pH7). This was prepared as a twenty times concentrated stock (20xssc = 3M sodium chloride, 0.3M trisodium citrate) 6xssc, 2xssc and 0.1xssc were 0.3, 0.1 and 0.005 dilutions respectively of 20xssc.

5xligase buffer consisted of 400mM tris-HCl pH7.5, 50mM $MgCl_2$, 10mM dithiothreitol, 0.33M A.T.P. The magnesium chloride and tris-HCl solutions were autoclaved as 1M solutions before appropriate dilution.

Gel loading beads were made as follows: 0.2% agarose in an aqueous solution of 20% glycerol, 10mM tris-HCl pH8.0, 10mM EDTA pH8.0, 0.1% bromophenol blue was autoclaved for five minutes and after the agarose had cooled to a gel the mixture was passed through a fine syringe to form a slurry.

Deproteinising mixture was a solution of hydroxyquinoline (0.05%) in a mixture of phenol (10 parts v/v), chloroform (10 parts v/v) and iso-amyl alcohol (3 methyl butanol) (0.4 parts v/v). The whole was equilibrated with a suitable buffer.

Scintillation fluid was a solution of P.P.O. (2,5, Diphenyloxazole, 0.5%) and P.O.P.O.P. (1,4,-Di-[2-(5-phenyloxazolyl)]-benzene, 0.03%) in toluene.

Diphenylamine reagent was made upon the day of use, by adding 300mg of diphenylamine and 300 µl of conc. sulphuric acid to 20ml of glacial acetic acid. 2 volumes of reagent were added to one volume of sample in 0.5M perchloric acid and 0.02 volumes of 1.6 $mgml^{-1}$ aqueous acetaldehyde added to the final mixture. Diphenylamine was recrystallised before use and batches of acetic acid were tested prior to use.

D. MATERIALS

Sources

Common laboratory chemicals (analytical grade) were from Fisons and biochemicals were from Sigma with the exceptions listed below.

Radiochemicals were from the Radiochemical Centre or from I.C.N. Pharmaceuticals.

DNase I (electrophoretically purified) was from Worthington.

DNA polymerase I and proteinase K were from Boehringer-Mannheim.

DNA ligase and EcoR1 endonuclease were from Miles. (Later batches of EcoR1 were from Porton Down).

POPOP was from Koch-Light.

Ficoll was from Pharmacia.

Diphenylamine (BDH) was recrystallised from 70% ethanol before use.

Nitrocellulose filters for hybridisation (as 2.5cm circles or large rolls) were from Sartorius (grade SM11306).

Batches of formamide (Fisons analytical grade) were tested and kept purely for hybridisation. Some batches gave a high pH due to the presence of ammonia.

2.II. METHODS

A. CONSTRUCTION AND HANDLING OF STRAINS

Construction of LE501

A culture of P162-8 was grown in nutrient broth overnight at 37°C without shaking, sedimented and resuspended to A_{450} of 0.9 in lambda buffer. The suspension was shaken at 37°C for 1hr. Aliquots (0.1ml) of this suspension and of 10^{-2} and 10^{-4} dilutions were plated in lambda soft agar containing thymine on lambda tryptone plates and when the soft agar had set, one loopful of a λcI857 Sam7 lysate was spread on the centre of each plate. The plates were incubated overnight at 37°C. The plate showing the clearest inhibition of growth in the region where the phage suspension was spread was taken, the soft agar from that region was excised with a sterile loop, added to a little sterile buffer and vortex mixed thoroughly. The resulting suspension was streaked out for single colonies, which were picked off with a sterile toothpick and tested for the genetic markers of P162-8. They were also tested for λ immunity by cross streaking against λcI and λvir.

Construction of recA strains

In the case of strains carrying no counterselectable markers in the recA region, thyA derivatives were selected by trimethoprim treatment as recommended by Miller (1972). Aliquots (0.1ml) of an overnight culture of each strain were spread onto M9 agar plates containing essential growth

requirements, thymine (50 μgml^{-1}) and trimethoprim (10 μgml^{-1}) and the plates incubated for 48hr. Colonies were purified on similar plates and then tested for genetic markers.

The recA56 marker was transferred into the auxotrophic strains from JC5088 as follows. Overnight broth cultures of the recipients and of the donor were diluted approximately 100 times into fresh broth and grown till the donor was at A_{450} of 0.15-0.20. 1ml of donor was mixed with 1ml of recipient and 1ml of fresh broth and the mixture grown for 30 min following which it was agitated vigorously using a vortex mixer for 2 min. Aliquots (0.1ml) were spread on suitable selective M9 agar plates to obtain thy^+ transconjugants (or $cysC^+$ or $pheA^+$ etc as appropriate), and incubated at 37°C for 2 days. The resulting colonies were picked off with sterile toothpicks and tested for nutritional requirements. U.V. sensitivity was also tested (Miller, 1972) by gridding in duplicate onto broth plates. One plate was irradiated with 200ergs mm^{-2} ($2x10^{-11}$ Jm^{-2}) and both were wrapped in foil and incubated for 6-8 hr at 37°C before scoring.

Strain storage

Strains were stored for long periods as 2ml glycerol suspensions (Miller, 1972). Bacteria were scraped off an agar plate with a sterile loop and resuspended in 1ml of broth. 1ml of 80% glycerol was then added and the whole mixed thoroughly and frozen at -20°C or -80°C. Glycerol suspensions of strains from the in vitro recombinant collection were stored as recommended by Feltham et al. (1978) in 2ml vials containing glass beads at -80°C.

Testing of Hfr strains

Rapid matings on agar plates were performed by method A of Low (1973) except that streptomycin for counterselection against KL99 was already present in the plates and counterselection against AB313 was by the omission of threonine and leucine from the mating plates.

Interrupted mating experiments were performed as recommended by Miller (1972). Fresh overnight cultures of KL99 and BB20 were diluted into fresh L broth supplemented with 0.2% glucose and grown to A_{450} 0.1, at which point 1ml of donor was mixed with 19ml of recipient in a pre-warmed flask and incubated at 37°C with gentle shaking. Samples were taken at 0, 5, 10, 15, 20 and 25 min after mating was begun, blended in a mating interruptor and plated on selective minimal agar plates lacking tryptophan, and containing streptomycin.

Testing of phage MS2 sensitivity was as follows. Part of each colony to be tested was inoculated into a separate 5ml test tube containing 1ml of L broth supplemented with 0.2% glucose and incubated for 2-4hr till moderately turbid (i.e. exponential growth phase). 0.1ml of MS2 suspension was streaked across an MS2 plate prewarmed at 37°C and allowed to dry. One loopful of each of the test and control cultures was streaked across the plate at a right angle to the 'phage streak. The plate was incubated at 37°C and inspected after 3-5hr and after over-night incubation.

B. PRODUCTION OF IN VITRO RECOMBINANTS

Restriction of DNA

The DNA samples (10 µg in 100 µl of 100mM tris-HCl pH7.5, 50mM NaCl, 1mM EDTA) were heated at 65°C for 5 min to inactivate any nuclease present, placed in ice and $MgCl_2$ was added to a final concentration of 10mM followed by an excess (40-200 units) of EcoR1 endonuclease. The mixture was incubated for 1hr at 37°C and again subjected to heat shock to halt the reaction.

Ligation of DNA

The ligation conditions were based upon the recommendations of De Vries et al. (1976). Appropriate quantities (see section 3.II) of digested DNA solutions together with 20 µl of 500 $\mu g ml^{-1}$ autoclaved gelatin solution and 40 µl of 5 x ligase buffer were made up to a final volume of 200 µl with sterile distilled water, warmed at 37°C for 10 min then held at 0°C for 1hr. Commercial DNA ligase (1 µl) was then added and the mixture incubated at 16°C for 48hr. The solution was then dialysed against 10mM Tris-HCl, 1mM EDTA pH8 overnight (three changes of 1.5l).

Transformation of ligated DNA

The transformation procedure (R. Diaz, personal communication) was a slightly modified form of that described by Cohen et al. (1972). A 200ml culture of recipient bacteria was grown in L broth to A_{450} of 0.8, chilled and harvested by centrifugation, washed in 100ml of 10mM NaCl

at 0°C and after re-sedimentation resuspended in 100ml of ice cold 30mM $CaCl_2$. The cells were kept in this suspension at 0°C for 20 min, sedimented and resuspended in 10ml of ice cold 50mM $CaCl_2$, then sedimented once again and finally resuspended in 1ml of 50mM $CaCl_2$. This suspension of competent cells was used immediately or stored in ice for upto 2hrs.

An appropriate volume of ice cold ligated DNA solution was mixed with 0.1 volumes of 0.5M $CaCl_2$ in a chilled 1.5ml polypropylene tube. A suspension of competent cells (450 ul) was added to the DNA using chilled sterile automatic pipette tips. The solutions were mixed thoroughly and left in an ice-bath for 1hr before being heated at 42°C for 2.5 min and transferred back to 0°C. The mixture was then diluted into 60ml of L broth and grown at 37°C with shaking for 90 min before the cells were sedimented, resuspended in 20ml of broth and plated out or frozen in glycerol as indicated in the text. With the exception of the ligated DNA solution all solutions were autoclaved before use.

Direct selection of recombinant clones

Aliquots (0.1ml) were spread onto M9 agar plates lacking a particular nutritional requirement (or having an appropriate carbohydrate as sole energy source) in order to select those bacteria in which the transforming plasmid complemented a particular genetic lesion. The M9 plates were also supplemented with ampicillin (50 $\mu g ml^{-1}$).

Indirect selection of recombinant clones

Transformants were selected by spreading 0.1ml aliquots onto nutrient agar (or Lagar) plates containing 30 $\mu g ml^{-1}$ of ampicillin trihydrate. After 24hr incubation the colonies were picked off with sterile

toothpicks and gridded (104 per plate) onto similar plates (master plates). A sub-master plate produced by re-streaking patches from a master plate was grown for several hours at 37°C to produce visible patches and replica plated with a velvet pad onto two similar broth-ampicillin plates. The first replica of the pair was carefully overlaid with 0.1ml of a fresh overnight culture of LE405 in 4ml of soft nutrient agar, without disturbing the newly replicated patches. Both plates were inspected after 5-8hr incubation at 37°C and again after further incubation overnight. Patches which did not produce areas of clearing (by colicin) in the lawn of indicator bacteria but which grew well on the control plate (growth on the test plate often being obscured by indicator growth) were re-gridded for further testing.

Direct complementation test of colicin non-producing clones

Broth-ampicillin sub-master plates were produced as described in the previous section. Each was then replica plated onto a range of selective M9 agar plates each lacking an essential growth requirement of LE500 or containing as sole carbon source a carbohydrate that LE500 could not utilise. The replica plates were inspected after 24 and 48hrs incubation. Tetrazolium indicator plates were used instead of or in addition to M9 plates for testing sugar markers.

Indirect complementation test of colicin non-producing clones

Complementation of auxotrophic markers carried by strains other than LE500 was tested by print matings, treating the *in vitro* recombinant strains as male donors and replica plating them onto a lawn of

auxotrophic recipient spread on M9 selective plates. Method A of Low (1973) was followed exactly except that the F^- recipient culture was spread carefully and evenly with a glass spreader instead of being flood seeded. Counterselection was by the omission of appropriate donor nutritional requirements (at least two in each case) from the transconjugant plates. Ampicillin (50 $\mu g ml^{-1}$) was sometimes added to the transconjugant plates (see section 3.II).

Characterisation of recombinant plasmid DNA

Cultures (200ml in M9 casamino acids medium) were amplified and lysed as described below for the preparation of plasmid DNA except that the cultures were labelled by the addition of deoxyadenosine (1mM) and ^3H thymine (1 $\mu g ml^{-1}$, 0.25 $\mu Ci ml^{-1}$). Only one caesium chloride-ethidium bromide density gradient purification was performed and the gradients were fractionated by taking 20 drop samples from the bottom of the tube. Samples (5 µl) of each fraction were applied to pencil labelled Whatman No.1 filters and prepared for counting as described by Clewell and Helinski (1970) : by immersing sequentially in cold 10% trichloroacetic acid, ethanol and ether. After scintillation counting the plasmid containing fractions from each gradient were pooled, extracted with propan-2-ol and dialysed as described below.

The DNA samples (50 µl) in a final mixture (100 µl) of 100mM Tris-HCl pH7.5, 50mM NaCl, 10mM $MgCl_2$ were incubated as described above for the restriction of DNA using 10 units of EcoR1 endonuclease. Samples (10 µl or 5 µl) were mixed with an equal volume of loading beads and loaded into the slots of a horizontal agarose gel. Samples were

electrophoresed at constant voltage in tris-borate or tris-acetate buffer. EcoR1 endonuclease digested λcI857 Sam7 DNA was used as a molecular weight marker.

Test of transforming properties of recombinant plasmids

Transformations were performed by the method described earlier, using 10 µl (0.2-2.0 µg) of plasmid DNA in 100 µl of 50mM $CaCl_2$, 10mM tris-HCl pH7.5 and 200 µl of competent cells. After outgrowth in broth, samples or dilutions were spread directly onto minimal selective plates and broth-ampicillin plates. Each transformant was then tested further for ampicillin resistance, and complementation of appropriate chromosomal lesions. Tests of MS2 sensitivity have been described earlier in this section. Colicin sensitivity of transformants was tested as follows. LE404 was streaked across the centre of a broth-ampicillin plate and grown for at least 24hr. The growth was then carefully removed with a spatula and the plate inverted for 15 min over a pad of tissue soaked in chloroform. The plate was then propped up at an angle to allow the chloroform vapour to disperse for a further 15 min. Strains to be tested were then streaked across the line of the original bacterial line of growth and the plates incubated overnight at 37°C. LE404 and LE405 were used as the colicin immune and colicin sensitive control strains respectively. An alternative method was to streak 0.1ml of concentrated colicin solution across the plate and to streak the test strains across the line of this. (The colicin solution was generously provided by J. Broome-Smith).

C. PURIFICATION OF DNA

Preparation of λ cI857 Sam7 DNA

The method used was based on that of Bøvre and Szybalski (1971). An overnight culture of LE501 was diluted into ten 100ml batches of tryptone broth supplemented with thiamine (10 μgml^{-1}) and thymine (2 μgml^{-1} if ^{3}H thymine - otherwise 10 μgml^{-1}), and these fresh cultures were grown at 30°C with shaking. At A_{450} 0.4 the cultures were moved to a shaking 42°C water bath for 30 min and then to a shaking 37°C bath. After growth for a further 3-4hr the cells were sedimented at 4500 rpm in a Sorvall GSA rotor for 10 min at 4°C, washed in lambda buffer and after resedimentation, resuspended in 20ml of fresh lambda buffer. Chloroform, (0.2ml) was added to lyse the cells and mixed thoroughly by vigorous agitation on a vortex mixer till thoroughly dispersed. After 12hr at 4°C the lysate was centrifuged at 7000 rpm for 20 min in a Sorvall SS34 rotor and the supernatant carefully decanted. Sufficient saturated caesium chloride solution was added to the supernatant to produce a refractive index of 1.3810, and the 'phage particles were banded by centrifugation in a Beckman 50Ti rotor at 32.5 krpm for 24hr at 7.5°C. The 'phage bands were removed through the sides of the transparent cellulose nitrate tubes with a hypodermic syringe, pooled in a fresh tube and the gradient centrifugation repeated. DNA was extracted exactly as described by Bøvre and Szybalski (1971). Basically the method consisted of dialysis against 10mM phosphate buffer pH7.5, heating at 60°C in 0.25% sodium dodecyl sulphate (S.D.S.) and extraction with phenol (3 times 1 volume).

Preparation of plasmid DNA

Strains bearing amplifiable plasmids (i.e. all those plasmids used except for pLG2) were grown to A_{450} 0.5 in one litre of M9 casamino acids medium, at which point chloramphenicol (200 $\mu g ml^{-1}$ final concentration) was added and the cultures were incubated for 14-16hr to amplify the plasmid (Clewell, 1972). pLG2 was prepared from an overnight culture (21, A_{450} 1.5) grown in M9 medium supplemented with essential growth requirements and ampicillin (50 $\mu g ml^{-1}$).

Routinely a cleared lysate procedure based on that of Katz et al. (1973) was employed. The bacterial cells were sedimented, washed, and finally resuspended at a maximum concentration of 200 A_{450} units ml^{-1} in 25% sucrose 50mM tris pH8.0 on ice. For each 3ml of resuspended cells, 1ml of a 4 $mg ml^{-1}$ solution of lysozyme in 0.2M tris pH8.0 was added. The mixture was left for 15-30 min at 0°C and the suspension examined microscopically to ensure that spheroplast formation had occurred. If no spheroplasts had formed the suspension was warmed at 37°C for 5 min then returned to the ice bath. After spheroplast formation, lysis was achieved by adding 1ml of 0.2M EDTA pH8 and, after a further 10 min at 0°C, 5ml of Triton X100 solution (2% in 50mM tris, 62.5mM EDTA pH8.0). The solutions were mixed well and the viscous mixture left in ice for 20 min, then centrifuged at 4°C for 20 min at 40,000g (18,000 rpm in a Sorvall SS34 rotor) and the supernatant carefully decanted and saved.

Strain LE541 lysed very easily and would not produce good cleared lysates so a "Hirt supernatant" (Hirt, 1967) was produced exactly as described by Katz et al. (1977).

DNA was precipitated from the cleared lysates by the method of Humphreys et al. (1975). Polyethylene glycol 6000 (PEG) (0.28 volumes of a 50% solution) and sodium chloride (0.14 volumes of a 5M solution) were added and the mixture left to precipitate at 4°C (6hr or overnight). In the case of "Hirt supernatants" which contain 1M NaCl, 0.6 volumes of water and 0.4 volumes of 50% PEG were added to give 10% PEG, 0.5M NaCl. The precipitate was sedimented by centrifugation at 3000 rpm (not more) for 5 min in a Sorvall SS34 rotor and redissolved in a little dilute buffer (e.g. T.E. buffer). Saturated caesium chloride solution and ethidium bromide were added to give a final refractive index of 1.3900-1.3905 and an ethidium bromide concentration of 300 $\mu g ml^{-1}$ and the mixture centrifuged at 36 krpm in a Beckman type 65, 70 or 75Ti rotor for 40hr at 15°C in a transparent nitrocellulose or thin walled polypropylene tube. The visible plasmid band was removed with a hypodermic syringe through the side of the tube, extracted with 4 times 1 volume of propan-2-ol and dialysed overnight against 10mM tris-HCl 1mM EDTA pH7.5 (T.E. buffer) (3 changes of 1l).

Preparation of DNA from strains C600 and W3110

DNA for use in the production of in vitro recombinant plasmids and in tests of the hybridisation background level was isolated by a modification of Marmur's (1961) method. A fresh overnight culture in L broth (1l, A_{450} 2.5) was sedimented by spinning for 10 min at 5000 rpm in a Sorvall GSA rotor, washed in 50ml of 25% sucrose 50mM Tris pH8, sedimented again and finally resuspended in 48ml of sucrose/tris solution. Lysozyme (7ml of a 10 $mg ml^{-1}$ solution in 0.25M tris pH8.0) was added,

the mixture shaken gently at 37°C for 30 sec and left to stand for 5 min in ice. After the addition of 25ml of EDTA pH8.0 the mixture was again left at 0°C for 5 min. In order to achieve lysis 54ml of Triton X-100 solution (2% w/v in 50mM tris 62.5mM EDTA pH8.0) was added and the mixture left for 20 min at 0°C after thorough mixing. Sodium perchlorate (27ml of a 5M solution) was then added so that the mixture became clearer. The solution was deproteinised by shaking with 81ml of chloroform/ isoamylalcohol (24:1) on a mechanical wrist shaker for 30 min, and the mixture centrifuged for 5 min at 5000 rpm in a Sorvall GSA rotor. The upper layer was pipetted off, avoiding the interface material, two volumes of cold ethanol were added to it and the DNA was spooled out on a glass rod. All subsequent steps were as described by Marmur (1961). Briefly the method was as follows: the DNA was redissolved in 10ml of 0.1xSSC, the SSC concentration was made up to 1xSSC and the mixture was deproteinised repeatedly (about five times) with chloroform/isoamyl-alcohol until little material appeared at the interace upon centrifugation (4000 rpm for 5 min in a Sorvall SS34 rotor). The DNA was spooled out and redissolved as before and treated with ribonuclease (30 min at 37°C 50 $\mu g ml^{-1}$ ribonuclease). The solution was again made upto 1xSSC and repeatedly deproteinised (about 4 times) until no more protein was extracted. The DNA was again spooled out of ethanol and redissolved in 9ml of 0.1xSSC. 1ml of 3M sodium acetate, 1mM EDTA pH7 was added and the DNA was precipitated onto a magnetic flea by the dropwise addition of 5.4ml of propan-2-ol. The precipitate was washed successively in 70%, 80% and 90% ethanol and redissolved in an appropriate buffer (0.1xSSC or TE).

Preparation of DNA from strains KL99, KL1, A3 and $A3^+$. (Lysis III)

Samples of culture (100ml), were poured over 30ml of crushed M9 ice and 3ml of pyridine and stored on ice for upto 2hrs. The cells were centrifuged down, washed and finally resuspended in 2ml of 100mM tris-HCl 50mM EDTA pH8.0 (DNA buffer). The lysis procedure was basically as described by Louarn et al. (1974). Lysozyme (30 µl of a 10 $mgml^{-1}$ solution in DNA buffer) was added and the mixture was incubated for 5 min on ice and 1 min at 37°C, before returning to 0°C. Sarkosyl (20 µl of a 10% solution) was added to lyse the cells followed by 300 ul of ribonuclease solution (1 $mgml^{-1}$ in 0.16M NaCl, 0.32M sodium acetate pH4.6 previously boiled for 10 min). The mixture was incubated at 37°C for 90 min then 20 µl of proteinase K (20 $mgml^{-1}$) was added and the mixture incubated further for 2hr at 37°C. Finally caesium chloride and ethidium bromide were added directly to the lysate and the mixture centrifuged as described for plasmid DNA using a Beckman 50Ti rotor. After taking fractions from the bottom of the gradient, the chromosomal band could be seen both by the viscosity of the solution and by ultra violet fluorescence. The fractions containing chromosomal DNA from a single gradient were pooled and treated as for plasmid DNA.

Preparation of chromosomal EcoR1 fragment from pLG4

pLG4 plasmid DNA, digested with EcoR1 endonuclease (50 µg DNA in 100 µl of restriction buffer) was mixed with an equal volume of loading beads and loaded (2.5 µg per slot) into an 0.3% agarose gel and electrophoresed for 11hr at 40v. The gel was stained as described below and examined on a U.V. transilluminator. The upper argE fragment bands

were excised with a scalpel and placed in a siliconised glass tube. The agarose was macerated with a pipette, mixed with 2 volumes of 10mM $NaClO_4$, 10mM tris pH7.5 and allowed to stand with occasional mixing while the agarose dissolved (30-60 min). The solution was then diluted with an equal volume of water and passed under pressure through a $1cm^3$ volume of hydroxyapatite in a 1cm diameter water jacketed glass column. The column was washed with 10ml of 0.16M potassium phosphate buffer (pH6.8) at room temperature and another volume at 60°C. The DNA was then eluted in 5ml of 0.3M potassium phosphate buffer (pH6.8) at 60°C.

Preparation of low molecular weight salmon DNA

Commercial salmon DNA (Sigma) was dissolved at a concentration of 10 $mgml^{-1}$ in water and supplemented with EDTA (pH8) to a final concentration of 20mM and NaOH to 0.3M. The solution was then heated at 100°C for 20 min. and then neutralised with HCl following the addition of tris pH7.5 to a final concentration of 0.1M. The DNA solution was then extracted with deproteinising mixture and pipetted off after centrifugation and precipitated with 2 volumes of ethanol at -80°C for 1hr after adding sodium acetate to a final concentration of 0.2M. The precipitate was sedimented, washed in 70% ethanol and vacuum dried before being redissolved at 5-10 $mgml^{-1}$ in water.

D. HYBRIDISATION BY THE METHOD OF KOURILSKY et al. (1971)

Loading of filters

Loading of filters was according to Bird et al. (1972). DNA (2 μg for each filter to be loaded) in 3ml of 0.1xSSC was denatured by adding 0.45ml of 1M NaOH and leaving for 10 min at 0°C. The pH was adjusted to 7.0 and the sodium concentration to 1.17M by the addition of 0.45ml of 1M NaCl and 1.56ml of 20xSSC. The DNA concentration was adjusted to 0.4 $\mu g ml^{-1}$ by the addition of 6xSSC. For the purposes of constructing standard curves, two solutions were prepared, each corresponding to the DNA mixture needed to load filters at either end of the curve. These were mixed in appropriate proportions to produce the solutions for loading of the other filters.

The filters (2.5cm nitrocellulose) were soaked in 6xSSC after labelling with a pencil, then placed on a multifiltration apparatus. Each filter was washed with 5ml of 6xSSC and the flow rate adjusted to 3-4ml min^{-1}. 5ml of DNA solution was passed through each filter, followed by 50ml of 6xSSC. The filters were then dried on a tissue pad and baked in a hot air oven at 80°C for 2hr between two sheets of filter paper, and stored in a dessicator.

Labelling of probe DNA

DNA was labelled by nick translation. At first the method of Macgregor and Mizuno (1976) was followed exactly. Later the volume and concentrations of reactants were modified as described in section 3.I.

The final method adopted was as follows. The reaction mixture (1ml) contained 50mM tris-HCl pH7.9, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 50 μgml^{-1} bovine serum albumin. The DNA concentration was upto 200 μgml^{-1} in which case dATP, dCTP, dGTP and dTTP were at 40μM each. DNA concentrations varied from 200 μgml^{-1} to 25 μgml^{-1} in which case the nucleotide triphosphate concentrations were reduced proportionately.

^{3}H-thymidine deoxyribonucleoside triphosphate (1.2μCi per μg of DNA) in 50% ethanol was evaporated to dryness in a dessicator, the above reaction mixture added to it and the whole preincubated at 15°C for 15 min. The reaction was started by adding deoxyribonuclease I (10ng) and DNA polymerase I (30 units). The mixture was incubated for 75 min at 15°C and the reaction stopped by the addition of 50 μl of 20% SDS, 50 μl of 250mM EDTA and heating to 60°C for 3 min. The mixture was extracted three times with equal volumes of phenol, dialysed extensively against TE buffer and stored at -80°C.

Samples were taken to monitor the course of the reaction as follows. Duplicate 5 μl samples were taken into 0.9ml of water containing 50 μg of bovine serum albumin, 0.1ml of 100% trichloroacetic acid (TCA) was added and the mixture allowed to stand on ice for 20 min before being filtered through Whatman GF/C filters and washed with 15ml of 5% TCA. Duplicate control samples were dried directly onto filters. The filters were dried with a heat lamp and counted in a Packard TriCarb scintillation spectrometer using toluene based scintillation fluid.

Hybridisation

DNA hybridisation using small filters in the presence of formamide (Kourilsky et al., 1971) was performed as described by Louarn et al. (1974). Changes in the incubation temperature and washing procedure were made as described in section 3.III. The final method was as follows. ^{3}H-labelled probe DNA (0.2 µg per filter) in 1ml of 0.1xSSC was sonicated at 15-18 µm amplitude for 8x15 seconds with an M.S.E. 150 watt ultrasonic disintegrator fitted with a 3mm exponential probe. The DNA was denatured at 0°C by the addition of 0.15ml of 1M NaOH and the solution neutralised 10 min later with 0.15ml of 1M HCl. The solution was then diluted with 20xSSC and distilled water to give a DNA concentration of 0.8 $\mu g ml^{-1}$ and an SSC concentration of 4X. (The NaOH was treated as equivalent to 0.05ml of 20xSSC for this purpose). Two 100 µl standard samples were taken directly onto 2.5cm nitrocellulose filters. The DNA solution was then diluted with an equal volume of formamide to give 0.4 $\mu g ml^{-1}$ in 2xSSC, 50% formamide and kept in ice. The loaded filters which had been soaked in 2xSSC for at least 30 min were rolled up carefully and placed into 0.5cm glass tubes. The appropriate probe DNA solution (0.5 ml) was then added to each tube. The tubes were sealed against evaporation with a little paraffin and incubated for 96hr at 45°C in a polyethylene glycol 400 bath. The filters were then pooled in a beaker and given two 20 min washes in 2xSSC 50% formamide at 40°C and three 20 min washes in 2xSSC at 40°C. The filters were then drained on a tissue pad, placed in glass scintillation vials and dried in an oven (80°C for 60 min), then counted using toluene scintillation fluid in a Packard Tri Carb scintillation spectrometer. Because the filters became rolled tightly

during the hybridisation procedure, the samples were counted for five changer cycles of 2 min rather than 1 cycle of 10 min counts. This allowed the orientation of the vial within the counter to be changed and therefore minimised the effect of that orientation on the reproducibility of counts. All tubes and flasks used for DNA solutions were cleaned in chromic acid and siliconised.

E. HYBRIDISATION BY THE METHOD OF SOUTHERN (1975)

Preparation of filters

DNA samples were digested as described previously but with a small (2-3x) excess of EcoR1 endonuclease, which had been previously titrated against the same lot of DNA. The DNA samples were mixed with two volumes of loading beads and left for several minutes before loading into a horizontal agarose slab gel. The gel was electrophoresed as indicated (normally 0.5% agarose at 40hr for 15hr) using a tris-borate (TB) buffer system. The gel was stained by soaking for 15 min at room temperature with occasional gentle shaking in 200ml of TB buffer supplemented with 0.4 $\mu g ml^{-1}$ of ethidium bromide. The gel was then photographed by virtue of fluoresence induced by an ultraviolet transilluminator. The gel was then soaked for 30 min in gel denaturing buffer and for 40 min in neutralising buffer.

Transfer of the DNA to a nitrocellulose filter was by use of a modification (A.J. Jeffreys, personal communication) of the apparatus of Southern (1975). A raised platform was covered with Whatman 3MM paper, the ends of which were immersed in 20XSSC to act as wicks. A sheet of

sellophane was placed over this and a hole cut in it slightly smaller than the gel. The gel was then positioned on top of this with care to avoid air bubbles between it and the Whatman paper. One sheet of nitrocellulose membrane was cut to a size just larger than the gel, wetted in 3XSSC and placed over the gel. One similarly sized sheet of Whatman 3MM paper which had also been soaked in 3XSSC was placed over the nitrocellulose filter, followed by five dry sheets. A pad of paper towels or disposable nappies was placed on top of that, followed by a glass plate and a 1kg weight. The apparatus was left at least overnight at 4°C until the absorbent pad became soaked. Following transfer the apparatus was carefully dismantled, the membrane filter marked with a pencil to indicate the position and orientation of the gel, washed briefly in 3XSSC and baked at 80°C for 2hr before being stored in a dessicator.

Labelling of probe DNA

The method used for the labelling of probe DNA with ^{32}P by nick translation (A.J. Jeffreys personal communication) was basically that of Jeffreys and Flavell (1977). 20μCi of α^{32}PdCTP, α^{32}PdGTP, α^{32}PdATP or α^{32}PdTTP (0.35-7.5KCi mmol^{-1}) in 50% ethanol was evaporated to dryness in a 1.5ml polypropylene tube inside a dessicator and redissolved in 33μl reaction mixture containing each of the other three nucleotide triphosphates (unlabelled) at a final concentration of 4μM each in 50mM tris pH7.9, 5mM $MgCl_2$ and 10mM 2-mercaptoethanol. The reaction was started by the addition of DNase I (5.5pg) and DNA polymerase I (2 units) and the mixture was incubated at 16°C for 90 min. The reaction was halted by the addition of 2.5μl of 20% SDS, 5μl of 0.25M

EDTA pH8.0 and 100µl of 10mM tris-HCl pH7.5. The mixture was extracted with 100µl of deproteination mixture, the non aqueous phase being re-extracted several times with 100µl aliquots of 10mM tris-HCl, 1mM EDTA pH7.5. The pooled aqueous phases were chromatographed in TE buffer on a 2ml Sephadex G50 fine column made in a siliconised pasteur pipette plugged with glass wool. 0.2ml fractions were collected in 1.5ml polypropylene tubes and monitored for Cerenkov radiation. The excluded fractions, which normally contained 40% of the label were pooled and frozen at -20°C for use the next day.

Hybridisation

Hybridisation with filters produced by the "Southern blot" procedure was by one of two methods. The original method (Jeffreys and Flavell, 1977) without dextran sulphate was as follows. The baked filters were cut into strips 9cmx3.5cm, having been marked to allow reconstruction of the whole. The strips were then sandwiched between two blank filters and transferred sequentially into a series of solutions in sealed perspex boxes at 65°C. Successive incubations were:-

(1) 30 min in 30ml of 3xSSC, (2) 3hr in 30ml of 3xSSC, 0.2% Ficoll, 0.2% bovine serum albumin (B.S.A), 0.2% polyvinylpyrollidone (P.V.P), (3) 1hr in 20ml of solution (2) supplemented with 0.1% SDS and 50 $\mu g ml^{-1}$ of low molecular weight denatured salmon DNA, (4) (the hybridisation step) overnight (16hr) in 20ml of solution (3) to which has been added the nick translated probe (heat denatured at 100°C for 5 min). (5) Six successive 5 min incubations in 50ml of 3XSSC, 0.2% Ficoll, 0.2% B.S.A, 0.2% P.V.P, 0.1% SDS, 50 $\mu g ml^{-1}$ salmon DNA. (6) Two 30 min washes in 0.1XSSC, 50

μgml^{-1} salmon DNA, 0.1% SDS. The strips were finally rinsed briefly in 3XSSC at room temperature and allowed to dry in a dessicator.

An alternative method (A.J. Jeffreys personal communication) which involved the use of dextran sulphate in the hybridisation mixture was used on one occasion. Modifications to the method of Jeffreys and Flavell (1977) described above were as follows. In steps (3) to (5) inclusive as described above 1XSSC was substituted for 3XSSC. Wash (2) was of only 2hr duration. An extra step (step (3a)) was inserted between steps (3) and (4). This new wash was identical to step (3) except that the solution was supplemented with 9% dextran sulphate. The actual hybridisation step was as before except that the solution was supplemented with 9% dextran sulphate and incubation was for 5hr only.

Autoradiography and counting

The hybridised strips of nitrocellulose were reassembled into the original full filter by taping carefully to a glass plate, and the glass plate was covered with clingfilm. The whole was autoradiographed at -80°C for an appropriate time using Kodak X-Omat film and an Ilford tungstate intensifying screen. The filter was marked with ^{35}S labelled ink prior to autoradiography to allow precise location and excision of the radioactive areas on the filter. A tracing of the autoradiograph was placed over the filter and the radioactive bands were cut from the filter by cutting through the tracing with a scalpel. The excised pieces of filter were placed in small plastic scintillation vials, dried at 80°C for 1hr, covered with 5ml of scintillation fluid and counted in a Packard Tri Carb scintillation spectrometer.

F. ESTIMATION OF DNA AND PULSE LABELLING

Measurement of DNA and cell mass

The cellular mass of cultures was estimated as A_{450} in a Gilford microsample spectrophotometer.

For the estimation of DNA content, samples of culture (50-75ml) were taken into an equal volume of ice-cold 15% trichloroacetic acid and kept for at least 90 min after which they were filtered onto Oxoid 4.7cm membrane filters. The filters were allowed to dry, immersed in a known volume of 0.5M perchloric acid and heated at 70°C for 30 min. The liquor was immediately decanted and assayed by the method of Burton (1956) except that the acetaldehyde was added last and the colour was measured as the difference between A_{595} and A_{700} as recommended by Giles and Myers (1965). Deoxyadenosine heated at 70°C in 0.5M perchloric acid was used as the standard.

The concentration of DNA purified for hybridisation was measured spectrophotometrically at 260nm wavelength.

Pulse labelling

Pulse labelling was performed by Dr E. Orr. The method basically was as follows. Duplicate samples of culture (0.5ml) were pulsed for 2 min using 2μCi ml^{-1} of ^{3}H thymidine (85 Ci $mmol^{-1}$) or 4μCi ml^{-1} of ^{3}H uridine. The pulses were stopped with 2ml of 10% trichloroacetic acid. Samples were left for 30 min in ice, filtered and washed with cold 5% trichloroacetic acid and 90°C distilled water before counting.

CHAPTER 3

3.I. MODIFICATION OF THE NICK TRANSLATION METHOD

The radioactive labelling of DNA to high specific activities *in vivo* is difficult and wasteful of materials. Methods for the labelling of very small quantities of DNA to extremely high specific activities *in vitro* have been available for some years (Nonoyama and Pagano, 1973; Commerford, 1971). Tests were therefore performed to see whether one such method, the nick translation procedure described by Macgregor and Mizuno (1976) could be used to label much larger quantities of DNA to moderately high specific activities using the isotope tritium. The long half-life of tritium would allow the DNA stocks so made, to be stored for long periods and to be used for many experiments for the sake of greater reproducibility.

The original protocol of Macgregor and Mizuno (1976) allowed one microgramme of DNA to be labelled in one hundred microlitres volume using four labelled nucleotide triphosphates. In a test of this method, the labelled triphosphates were replaced by the same concentrations of unlabelled triphosphates, and a small amount of ^{3}H-thymidine triphosphate was added in order to follow the course of the reaction. 'Phage lambda DNA and commercial salmon DNA were used for this, and subsequent tests. The results, shown in Fig. 3.1 show the expected efficiency of incorporation of label into acid precipitable material.

In two further tests, the DNA concentration and the concentrations of the nucleotide triphosphates were increased five-fold and the DNA polymerase concentration was progressively reduced. The results (Fig. 3.2)

Figure 3.1. "Nick translation" of DNA

DNA samples (1 μg) were nick translated using the method of Macgregor and Mizuno (1976). The DNA was incubated at 15°C in a 100 μl mixture using 10 $ngml^{-1}$ of DNAse 1 and 120 units ml^{-1} of DNA polymerase I. The method was otherwise as described in section 2.II. Samples were taken at the times indicated. The DNA used was lambda DNA (○) or commercial salmon DNA (●).

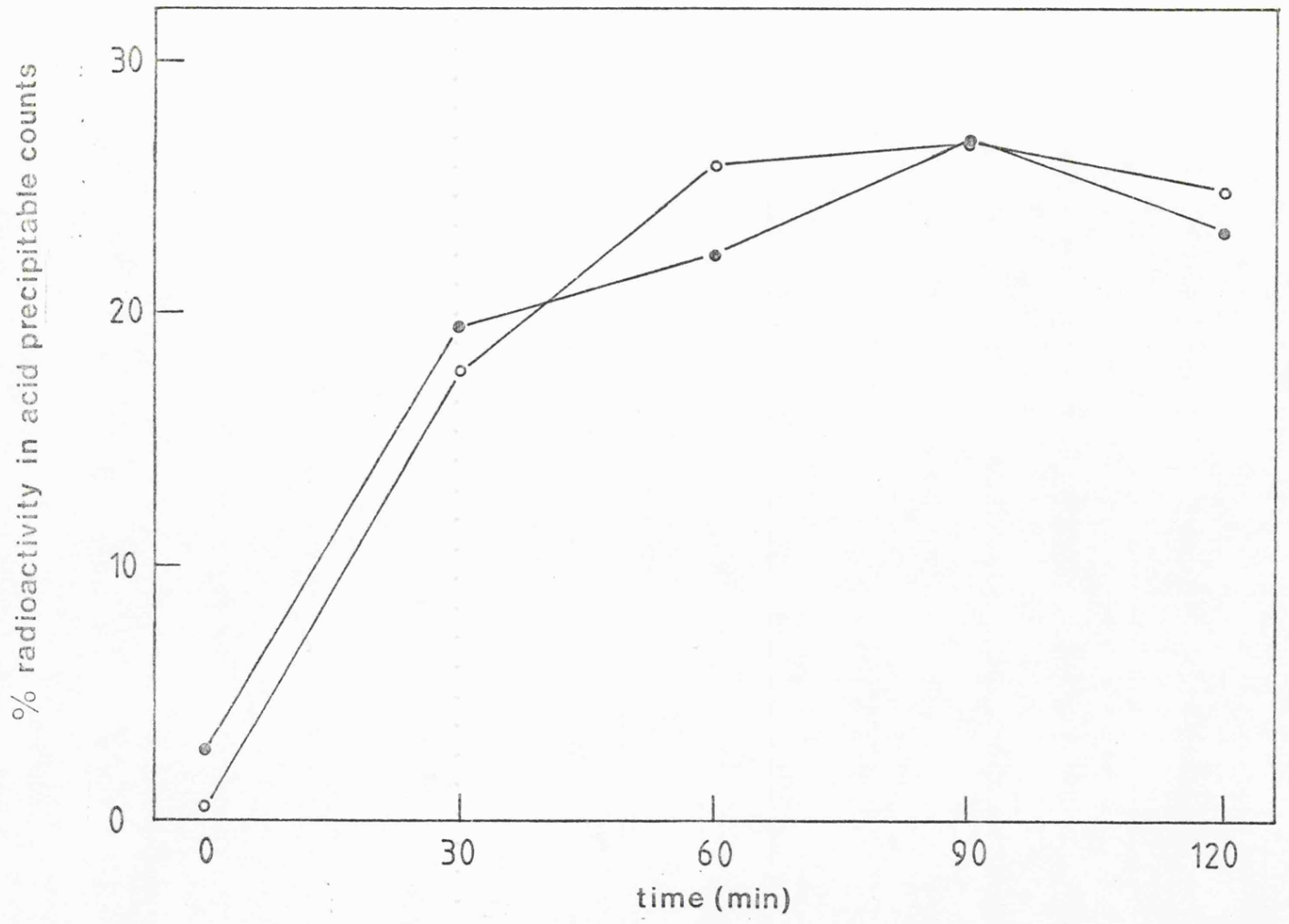
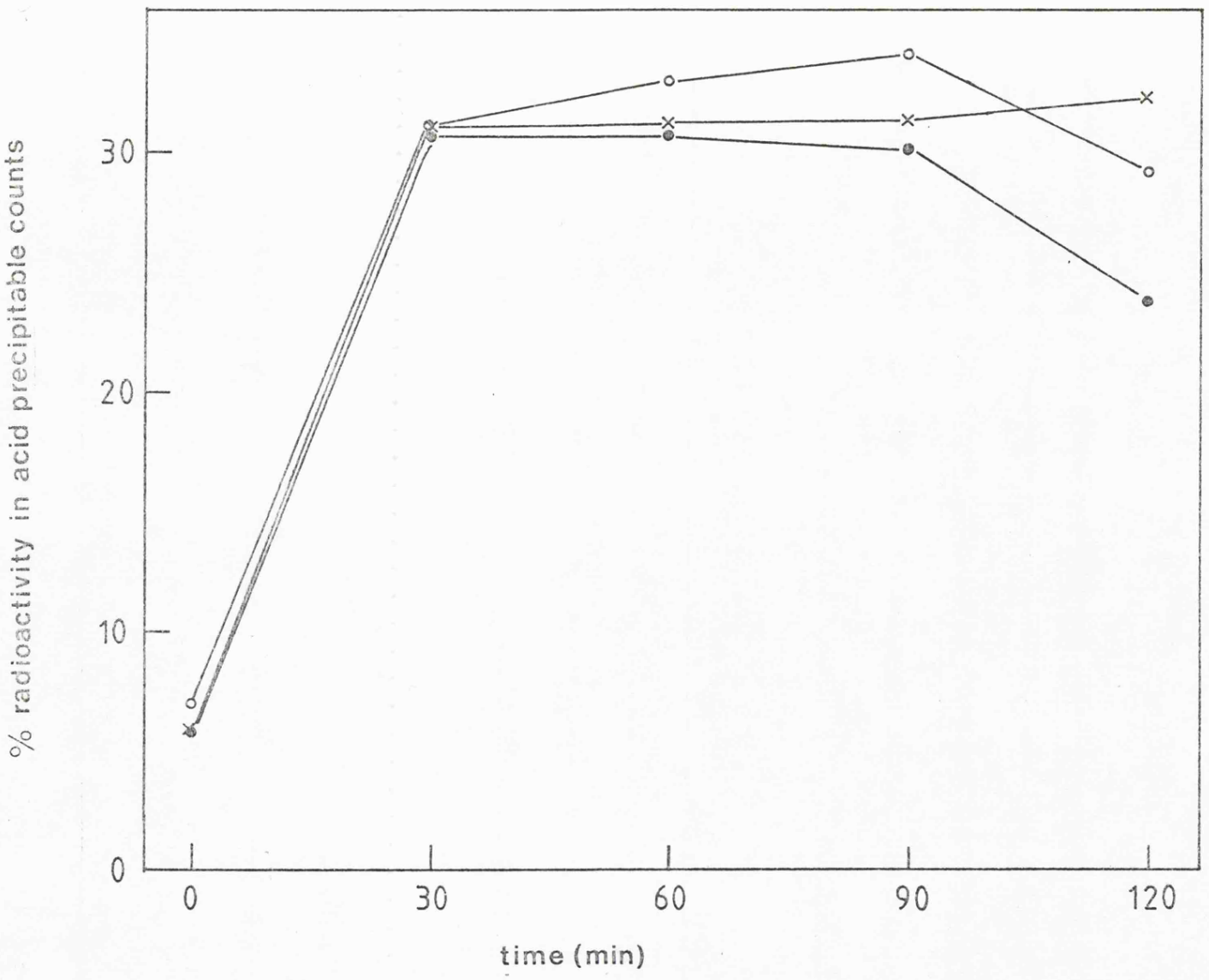

% radioactivity in acid precipitable counts
30
20
10
0
0
30
60
90
120
time (min)

Figure 3.2. Modification of "nick translation" of DNA

Samples of salmon DNA were incubated as described in the legend to Fig. 3.1 except that the DNA concentration was 50 $\mu g ml^{-1}$ and the DNA polymerase I concentration was either 120 units ml^{-1} as before (o), 60 units ml^{-1} (●) or 30 units ml^{-1} (×).


% radioactivity in acid precipitable counts
0
10
20
30
0
30
60
90
120
time (min)

show that the polymerase concentration could be reduced from 120 units ml^{-1} to 30 units ml^{-1} without affecting adversely, the kinetics of the reaction, or the final level of incorporation of label into the DNA. Further reductions in the polymerase concentration (Fig. 3.3) were deleterious.

Finally the method was tested again using a new batch of lambda DNA. Although this new batch regularly gave poorer incorporation the results were acceptable and it was shown that the concentrations of the DNA and of the triphosphates could be proportionately further increased to 200 $\mu g ml^{-1}$ of DNA (Fig. 3.4).

Concentrations of 25 $\mu g ml^{-1}$ to 200 $\mu g ml^{-1}$ of DNA were used for routine labelling of DNA. Although the ^{3}H-thymidine triphosphate was added as a 50% ethanol solution in the above experiments and up to 3% ethanol concentration in the final reaction mixture could be accommodated without ill effect, in later routine labelling procedures the ^{3}H-thymidine triphosphate solution was evaporated to dryness in a dessicator and redissolved in the final reaction mixture prior to the addition of enzyme. During the routine labelling of plasmid DNA the level of incorporation of label was between 15% and 45% after 75 minutes, when the reaction was halted, except for one sample which only showed 6% incorporation. These labelled DNA samples were shown to be digested normally with EcoR1 endonuclease, giving the usual clear bands upon agarose gel electrophoresis.

Figure 3.3. Second modification of "nick translation" conditions

The experiment was as that described in Fig. 3.2 except that the DNA polymerase concentrations were further reduced to either 30 units ml^{-1} (o), 10 units ml^{-1} (×) or 2 units ml^{-1} (●).

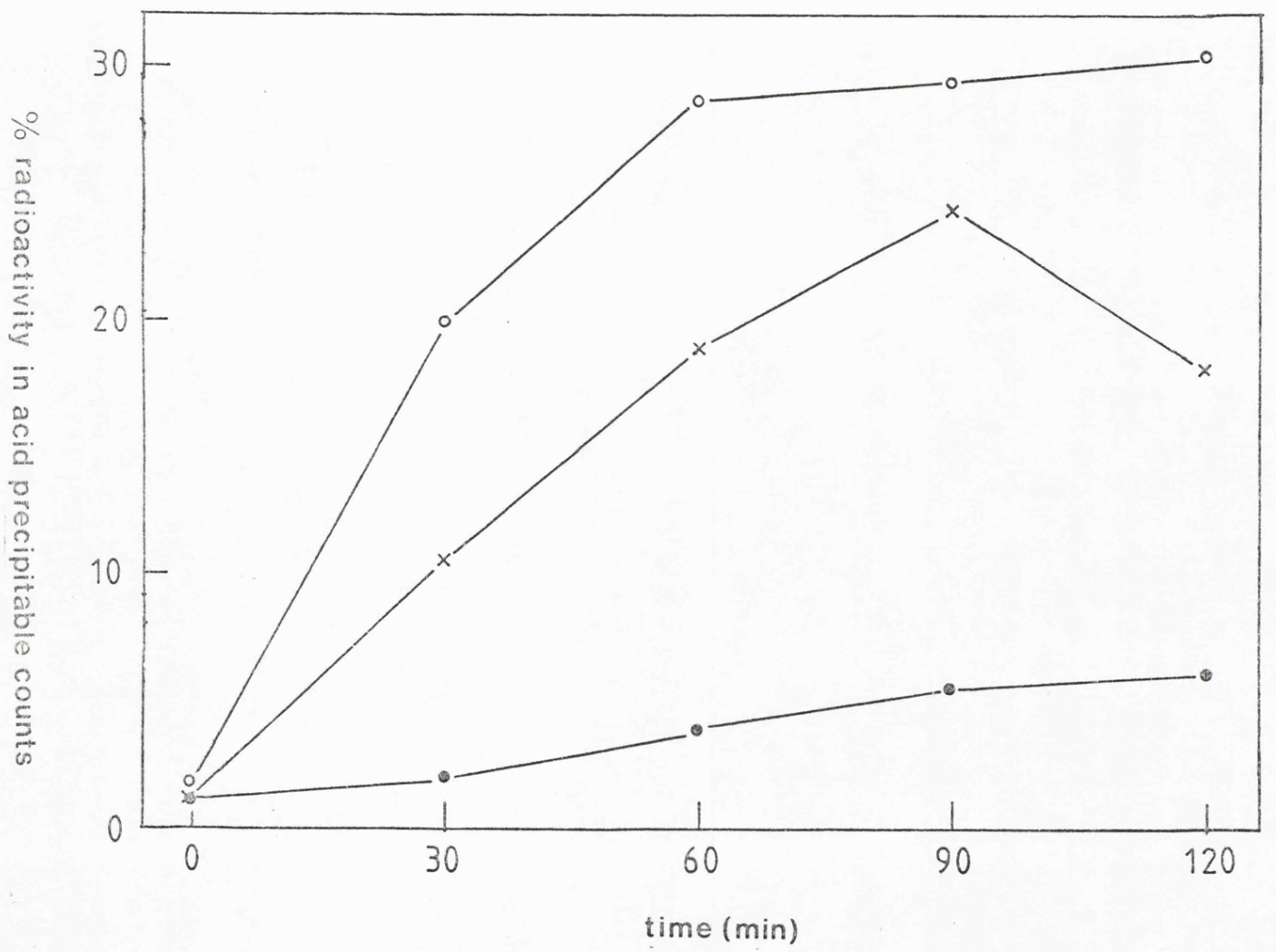
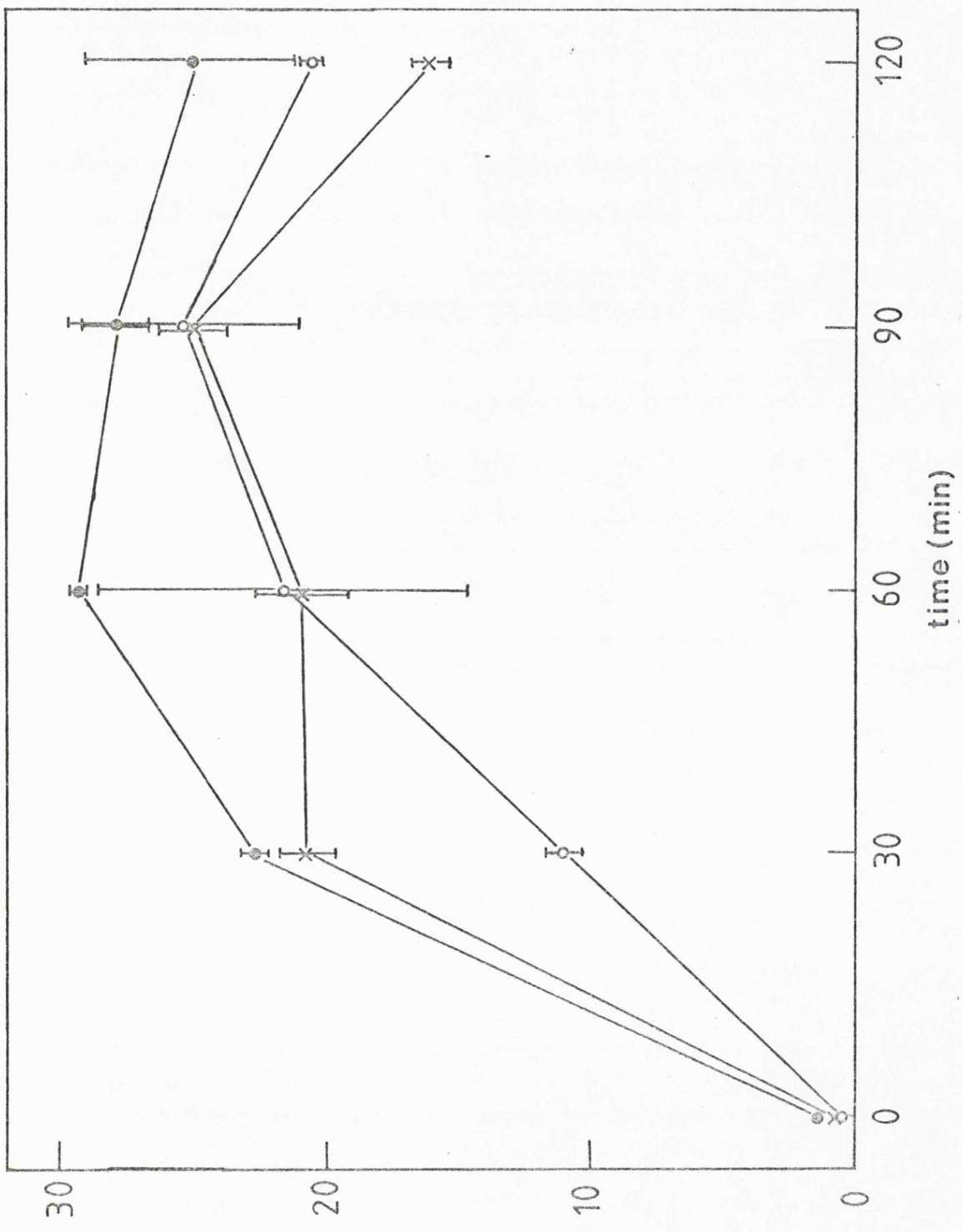

30
20
10
0
% radioactivity in acid precipitable counts
0
30
60
90
120
time (min)

Figure 3.4. Final modification of "nick translation" conditions

Samples of DNA were incubated exactly as described in section 2.II in a 1ml reaction mixture containing DNA polymerase at 30 units ml^{-1} and either salmon DNA at 50 $\mu g ml^{-1}$ (●), lambda DNA at 50 $\mu g ml^{-1}$ (✕) or lambda DNA at 200 $\mu g ml^{-1}$ (○). Points represent a mean of two readings with the standard error of the mean shown as error bars.


0
30
60
90
120
time (min)
0
10
20
30
% radioactivity in acid precipitable counts

3.II. CONSTRUCTION OF A RECOMBINANT PLASMID COLLECTION

In order to obtain a set of homologous hybridisation probes, an attempt was made to construct *in vitro* a collection of plasmids, each carrying a fragment of *E.coli* chromosomal DNA, the whole collection being representative of every region of the *E.coli* chromosome. RSF2124 (So *et al.*, 1975) was chosen as the cloning vector. This plasmid consists of the small plasmid ColE1 containing a transposon (TnA) coding for resistance to ampicillin (*bla*$^+$). ColE1 has been used as a cloning vehicle because it has a single recognition site for the restriction endonuclease EcoR1 located within the gene coding for the production of colicin E1 so that insertion of foreign DNA into this site leads to inactivation of the colicin gene (Hershfield *et al.*, 1974). Thus cells carrying RSF2124 may be selected by virtue of their resistance to ampicillin. Those which are carrying *in vitro* recombinant derivatives may then be distinguished by their inability to produce colicin E1.

Accordingly 6 µg of *E.coli* W3110 DNA restricted to the limit with EcoR1 as described in section 2.II was ligated to 4 ug of RSF2124 similarly restricted. The whole of the ligated DNA (150 µl) was transformed into *E.coli* strain LE500 and the transformed cells grown out in 60 ml of broth. After sedimentation and resuspension in 20 ml of broth, part of the culture was spread in 0.1 ml aliquots onto broth-ampicillin plates and the rest frozen in 40% glycerol to be plated out later.

The ampicillin resistant clones were then tested for the ability to produce colicin. The original method adopted was to replica plate the colonies onto broth-ampicillin plates and grow overnight. One replica

was then chloroformed and overlaid with a lawn of LE405 in soft agar. This unfortunately produced large areas of killing which were in places confluent, making it impossible to distinguish colicin non-producing colonies. A second method (J. Broome-Smith personal communication) was therefore tested and after some experiment the procedure described in section 2.II was adopted. This method allowed equal spacing of the clones on the plate and produced much smaller areas of killing.

Two points should be noted here. The very great production of colicin observed with the former method is not normally seen with ColE1 plasmid-containing strains (J. Broome-Smith personal communication) but it has since been noted during the routine testing of RSF2124-containing strains, that this massive production of colicin occurs only on plates containing ampicillin. Secondly, it was clearly seen that isolates later identified as colicin non-producers usually grew more strongly on broth-ampicillin plates than those identified as colicin producing. It was found that about 80-90% of the colicin non-producing strains could be identified in advance by the strength of their growth. In almost every case strongly growing clones were found not to produce colicin. Vigour may therefore prove to be a useful characteristic for rapid screening in future cloning experiments, though for the purposes of this study the results of the soft agar overlay test were always taken to be definitive.

728 colicin non-producing clones were selected for further screening and designated LE10001-LE10728. These were tested by replica plating for complementation of the LE500 markers *thr* *leuB* *his* *argE* *galK* *ara* *mtl* and *xyl* and by cross streaking against phage for *tsx*. All were found to have the phenotype of LE500 except for three clones which were weakly *thr*$^+$ (LE10129, LE10378 and LE10525) and one which was *mtl*$^+$ (LE10214).

Further screening of the colony collection was performed by means of F'*lac*$^+$ mediated plasmid mobilisation. The RSF2124 recombinant clones were streaked out and mated with a number of F$^-$ auxotrophic strains by replica plating on selective plates as described (section 2.II) to screen for complementation of the various genetic lesions carried by the auxotrophs. In the first such experiment LE1001-LE10312 were screened for the presence of plasmids carrying the genes *man*$^+$, *aroD*$^+$, *pyrD*$^+$, *trp*$^+$, *purE*$^+$, *pheA*$^+$, *argA*$^+$, *cysC*$^+$ and *lysA*$^+$ by mating them with GMS343 (*man*$^-$), GMS407 (*aroD*$^+$), KL188 (*pyrD*$^-$ *trp*$^-$), AT2092 (*purF*$^-$ *pheA*$^-$) and AT713 (*argA*$^-$ *cysC*$^-$ *lysA*$^-$) on appropriate selective media. The experiment was difficult to assess due to the large numbers of chromosomal recombinant colonies on all of the plates. In one case (*man*) there was confluent growth when one isolate (LE10268) was mated. This possible *man*$^+$ clone was kept for further investigation. Two other clones, LE10076 and LE10095 which seemed to transfer respectively *cysC*$^+$ and *pheA*$^+$ slightly more efficiently were also kept for further analysis.

A *rec*$^+$ strain had been chosen as the recipient for the original transformation since testing for colicin immunity, a useful confirmatory test for the presence of RSF2124, could not be easily performed on a *recA*$^-$ strain (R. Diaz personal communication). Unfortunately this led to a high level of chromosomal mobilisation in the above screening procedure. In an attempt to reduce this, *recA*$^-$ derivatives of the various F$^-$ strains were synthesised for use as recipients. This was done by selecting for *thyA* mutants where the strains did not already harbour markers in the *recA* region of the chromosome and then by selecting prototrophic recombinants in a mating with a *recA*$^-$ Hfr (JC5088) and screening as

described (section 2.II). In an experiment to screen clones as before but using *recA*$^-$ recipients the *purF* marker of LE521, the *lysA*, *malA* and *argA* markers of LE515 and the *argG* marker of JC1553 were screened using 312 of the clones. The background was reduced somewhat by the use of *recA*$^-$ recipients but again it was high enough to make the results equivocal even when in a repeat experiment, ampicillin was included in the selective plates. The markers *lysA* and *argG* gave a lower background level than the others and two possible *argG*$^+$ clones (LE10065 and LE10684) were kept for further testing.

It was nonetheless clear that a more direct method of screening was needed. A portion of the original transformed culture which had been frozen in glycerol was therefore plated out on selective minimal plates. The selections were for His$^+$ Gal$^+$ Ara$^+$ and Xyl$^+$. 30x0.1 ml portions were selected for His$^+$. The remaining 2 ml of culture was diluted into 20ml of broth, grown out overnight and plated out to select for the four markers. No colonies with the desired phenotypes were obtained.

A final attempt was made to select markers in those regions of the *E.coli* chromosome not already represented by identified clones: the 40-55 min region and the 10-30 min region on the linkage map (Bachmann and Low, 1980). A second restriction and ligation was therefore performed essentially as before, using 10 ug of *E.coli* W3110 DNA and 1.3 µg of RSF2124 DNA. 25 µl of the ligated DNA solution was used to transform each of three strains BB21, LE521 and LE543. The transformation was as described (section 2.II) except that the transformed cells were grown for 90 min in 200ml of broth before being centrifuged and resuspended in 20ml. In each case half of the culture was plated out directly and half frozen in glycerol. The BB21 transformants were plated for either *lacY*$^+$, *galK*$^+$,

trp$^+$ or *his*$^+$, the LE521 transformants for *malA*$^+$ or *purF*$^+$ and the LE543 transformants for *gltA*$^+$. One plate was used to estimate the number of Amp^R transformants for each strain. Only LE521 transformed well yielding ~10^3 Amp^R transformants in the first half of the culture. One strongly growing Pur$^+$ colony was tested and found to have the expected phenotype. This was designated LE562 and kept for further study. The frozen portion of the above cultures was diluted into broth, incubated for 2hr, centrifuged and resuspended in buffer before plating out as before. This time LE543 gave $2x10^3$ colonies, BB21 gave $3x10^3$ and LE521 $2x10^5$. It is not clear why the number rose so dramatically after only two hours growth as this cannot be accounted for in terms of growth of the culture. Presumably ninety minutes was insufficient time for expression of the *bla* gene after transformation. No further clones of the desired phenotype were found.

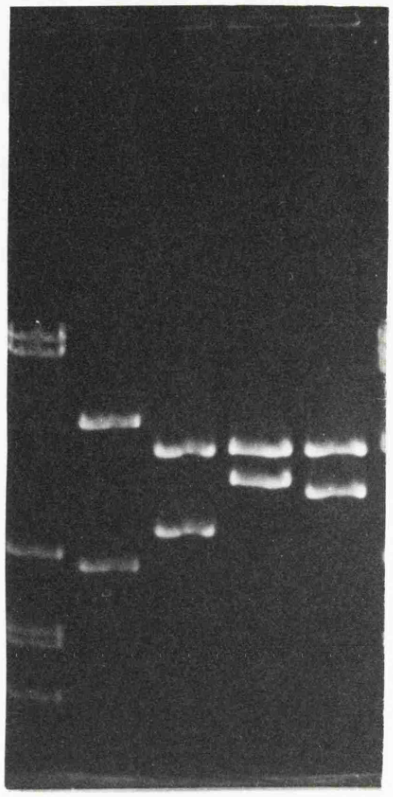
Plasmid DNA was prepared as described (section 2.II) from LE10076 (putative *cysC*$^+$ plasmid = pLG20), LE10095 (*pheA*$^+$? = pLG13), LE10214 (*mtl*$^+$? = pLG17) LE10268 (*man*$^+$? = pLG18) and LE562 (*purF*$^+$? = pLG19) for further testing. The restriction patterns are seen in Fig. 3.5. The last four mentioned each gave bands corresponding to the mobility of RSF2124 plus at least one band of chromosomal DNA. LE10095 gave two bands other than the vector. It is not clear whether this is due to the chromosomal DNA in the original experiment having been incompletely digested, due to two fragments from different parts of the chromosome having been ligated together into the plasmid molecule, or thirdly due to two separate recombinant plasmid molecules being present in the same cell. This plasmid was not used for any hybridisation experiments and

Figure 3.5. EcoR1 digested plasmids

The figure shows agarose gel electrophoresis patterns of the plasmids used as hybridisation probes and of other plasmids constructed during the course of this study. The samples were prepared for electrophoresis and run using a tris-borate system as described (section 2.II). The conditions were (1) and (2), 0.5% agarose 40v 15hr, (3) 0.5% agarose 40v 11hr, (4) 0.7% agarose 40v 11hr. All of the DNA samples were digested with EcoR1 endonuclease and were (a) λ size marker (fragment sizes) 15.7Md, 13.7Md, 4.7Md, 3.7Md, 3.5Md, 3.0Md, 2.0Md. (b) RSF2124 size marker 7.3Md. (c) pLG17. (d) pLG18. (e) pLG19. (f) pLG2. (g) pLG4. (h) pLG5 (partial digest). (i) pVH5. (j) pLG13. (k) pLG20.

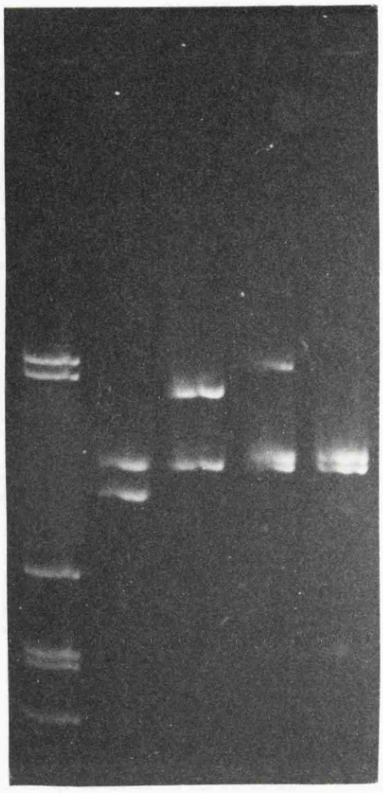
a f e c

1



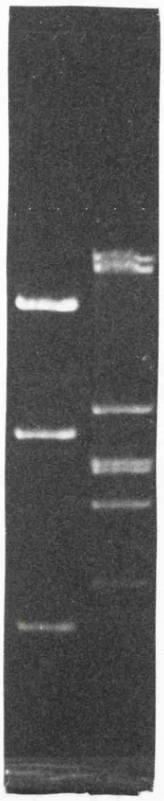
a d g h c

2



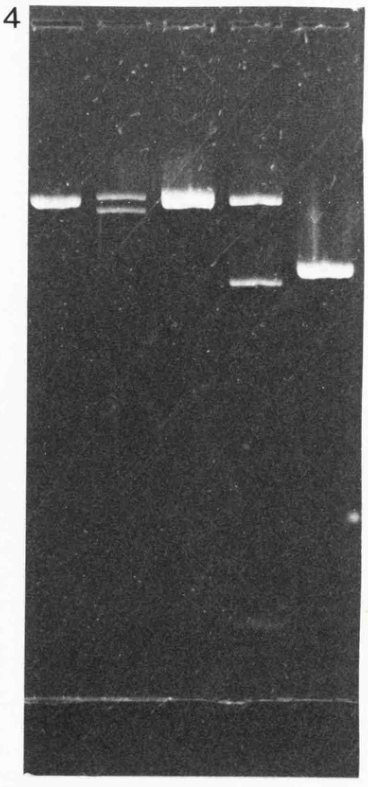
i a

3



b d c j k

4



no attempts were made to distinguish between these possibilities though it may have been possible to do so by the use of the "Southern blotting" technique (Southern, 1975). pLG20 was obviously anomalous yielding only one band of DNA which did not correspond to the linear form of RSF2124. This had most probably arisen by deletion of part of a larger recombinant plasmid but again the plasmid was not used for hybridisation and not tested further except by transformation.

The above mentioned plasmid DNA samples were tested for the ability to transform appropriate bacterial strains to Amp^R or to prototrophy for the genetic markers they were expected to complement. The colonies selected as Amp^R were tested for complementation of relevant auxotrophic lesions and colonies selected for prototrophy were tested for sensitivity to ampicillin. In the cases of pLG19 and pLG17, which were transformed into LE521 and AB2463 respectively, the result was clear cut. All Amp^R transformants were respectively Pur^+ or Mtl^+ and all Pur^+ or Mtl^+ transformants were Amp^R. pLG20 gave no Cys^+ transformants of AT713 and all of the Amp^R transformants were Cys^-. pLG13 and pLG18 transformed poorly. AT2092 and LE536 were the respective recipient strains. In both cases, colonies selected for the Amp^R phenotype had one of two different phenotypes with respect to the unselected marker. The Amp^R Phe^- or Amp^R Man^- colonies were probably chromosomal amp mutants. These "mutants" could be distinguished from the true Amp^R Phe^+ or Amp^R Man^+ colonies by their colonial form, since the latter gave rise to microcolonies in their immediate vicinity. This was presumably due to the secretion of β lactamase into the growth medium. These true transformants, unlike the other type, were also colicin immune, indicating the presence of a ColE1

derivative. Colonies selected as Man^+ were all Amp^R but large numbers of Phe^+ "revertants" made the direct selection of Phe^+ transformants difficult. pLG17, pLG18 and pLG19 were taken to be genuine recombinants between RSF2124 and a single EcoR1 fragment carrying respectively mtl^+, man^+ or $purF^+$. Strains transformed with pLG13, pLG17 and pLG18 respectively were shown to be F^- by a 'phage MS2 sensitivity test and were designated LE537, LE539 and LE541 was used for all subsequent preparations of pLG18 DNA.

During the screening outlined above, a number of plasmids suitable for use as a collection of hybridisation probes were obtained. Although some of these plasmids were not finally chosen for use in any experiments, most of the E.coli chromosome was represented in the collection when the plasmids pLG4 ($argE^+$) and pLG5 ($proA^+$) constructed by R. Diaz (personal communication) from the same vector and C600 DNA were included (see Tables 2.2 and 3.1). Two important gaps were filled by the use of other probes. These were pLG2 ($oriJ^+$), pVH5 (trp^+) and λ318 ($oriC^+$) (see Table 2.2). The last three replicons were constructed differently from the rest of the collection with heterologous vector DNA but all are known to contain only one fragment of chromosomal DNA, (Diaz and Pritchard, 1978; Diaz et al., 1979; Hershfield et al., 1974). Plasmids pLG2 and pLG6 (the latter being the source of the chromosomal fragment of λ318 (W. Brammar personal communication) were constructed by the same biochemical procedure as plasmids pLG4 and pLG5 and from the same stock of DNA (R. Diaz personal communication) through the selection procedures, and thus the vector DNA were necessarily different. The collection of probes used for hybridisation were therefore as similar in their properties as could be arranged.

Table 3.1. Summary of testing of plasmids constructed *in vitro*

Plasmid No.	Chromosomal markers complemented directly or in mating	Plasmid DNA present	Phenotype of transformants	No. of bands in agarose gel eleotrophoresis of *Eco*R1 digest	Homology with chromosomal DNA
pLG11	*argG*	-	-	-	-
pLG12	*argG*	-	-	-	-
pLG13	*pheA*	yes	Phe^+ Amp^R [b]	3	-
pLG14	*thr*	no [a]	-	-	-
pLG15	*thr*	-	-	-	-
pLG16	*thr*	-	-	-	-
pLG17	*mtl*	yes	Mtl^+ Amp^R	2	-
pLG18	*manA*	yes	Man^+ Amp^R	2	yes
pLG19	*purF*	yes	Pur^+ Amp^R	2	yes
pLG20	*cysC*	yes	Cys^- Amp^R	1	-

a no DNA was obtained from one attempted preparation

b when selected for amp^R (see section 3.I)

\- indicates not tested

The failure to obtain recombinant plasmids carrying among others the trp, gal or his operons as related above was most probably due to the inadequate number of clones screened though in the case of his and gal the probabilities of obtaining them from the numbers of clones screened was respectively 0.87 and 0.71. It is interesting to note that while all three have been rescued in vitro from EcoR1 limit digests of E.coli DNA (Clarke and Carbon, 1975; Collins et al., 1976a; Kozlov et al., 1977) the gal plasmid contained several chromosomal fragments. These may have been necessary for the expression of the gal operon. Hershfield et al. (1974) found that a fragment of 'phage DNA was necessary for the expression of the trp genes isolated on an EcoR1 fragment of transducing 'phage DNA. This is also a possible explanation for the presence of two fragments of chromosomal origin in pLG13, though no steps have been taken to confirm this.

DNA of the selected plasmids was prepared as described (section 2.II). Plasmid pLG18 DNA was prepared from LE541 (one of the transformants described above) to further ensure the genetic purity of the plasmid. The DNA finally used for hybridisation was restricted and tested on gels to confirm that the DNA was genetically pure as tested by a unique restriction pattern, and free from substantial traces of chromosomal DNA. After nick translation (see section 3.I) the DNA was normally tested again in a similar manner.

3.III. TESTING AND REFINEMENT OF THE HYBRIDISATION METHOD

As a previous study (Collins, 1971) has indicated that insufficient sonication of probe DNA can affect DNA annealing adversely, the effect of sonication amplitude on hybridisation was tested. No difference could be detected over the range tested (3μm - 17μm); 15μm to 17μm was therefore adopted routinely for subsequent experiments. New analytical grade formamide was found to be necessary for efficient hybridisation, being better than an old batch of analytical grade formamide and a batch of deionised formamide.

A potentially important disadvantage of using E.coli DNA fragments as hybridisation probes is that the background cannot be measured and corrected. The quantity measured by scintillation counting of a hybridisation filter consists of several components: (a) the general background due to environmental radiation (b) probe DNA sticking to the material of the filter itself (c) the true specific hybridisation of probe DNA to homologous sequences in the DNA bound to the filter, and (d) a fourth source due to probe DNA adhering non-specifically to non-homologous sequences or simply becoming entangled in the DNA on the filter. The first two components are small and easily controlled by "loading" blank filters with buffer containing no DNA and subjecting them to the hybridisation procedure. The last source of counts (d) which I will call "nonspecific background" cannot be directly measured and corrected. In the system of Bird et al. (1972) where 'phage DNA is used to probe lysogen DNA, non-lysogen DNA is the control but no such control is normally available where E.coli DNA fragments are used as probes. This would be a less serious problem if the nonspecific back-

ground were small in comparison to the specific hybridisation. An attempt was therefore made to estimate the level of the nonspecific background for the pLG4 (*argE*$^+$) probe. Unlabelled pLG4 DNA was digested with *EcoR1* endonuclease and the fragment of chromosomal origin was separated as described in section 2.II. A standard curve was then constructed by loading filters with 2ug of DNA extracted from a stationary culture of *E.coli* C600 and varying amounts of *argE*$^+$ *EcoR1* fragment DNA. Knowledge of the size of the *argE*$^+$ fragment and of the *E.coli* chromosome allowed the calculation of the number of *argE*$^+$ gene copies added as restriction fragment DNA in relation to the number already present in the chromosomal DNA. The number of counts expected to bind to a filter loaded with just *E.coli* DNA could therefore be calculated. Comparison of this calculated figure with the number of counts actually binding gave an estimate of the level of nonspecific hybridisation (Fig. 3.6).

In subsequent similar experiments, attempts were made to reduce the nonspecific background by adding various competitor DNAs and by increasing the stringency of the hybridisation and washing (Figs. 3.7 and 3.8). Competitor DNA seemed to have no significant effect confirming that the nonspecific background element was not true hybridisation. In particular the presence of competing RSF2124 sequences in the form of pLG5 (*proA*$^+$) did not reduce the nonspecific background showing that semi-specific hybridisation of vector sequences was not responsible for this element in the total count. The more stringent washing conditions did have an effect upon the nonspecific background. Although the indirectness of the method allows room for considerable errors in the estimation of the absolute level of the nonspecific background the relative reduction from

Figure 3.6. Standard curve hybridisation with pLG4 probe

The procedure for loading and hybridising the DNA was as described in section 2.II ("Kourilsky hybridisation") except that the hybridisation temperature was 42°C and the post-hybridisation washes were conducted at room temperature. The post-hybridisation washes were 30 min in 2xSSC, 50% formamide and four 20 min washes in 2xSSC.

All of the filters were loaded with 2 μg of DNA obtained from a stationary culture of E.coli C600. Varying quantities of the argE EcoR1 fragment from pLG4 were added to the filters to increase the relative gene dosage of the argE gene. To calculate relative gene dosages it was assumed that the relative gene dosage in the stationary culture was 1.0. The size of the E.coli chromosome was taken to be 2.5×10^9 daltons (Cooper and Helmstetter, 1968) and that of the E.coli argE EcoR1 fragment to be 1.28×10^7 daltons (Kozlov et al., 1977). Thus 10ng of argE fragment contains the same number of argE genes as 2 μg of E.coli DNA and should give the same signal.

The points represent counts of individual filters, corrected by subtracting the mean of the counts binding to three blank filters (i.e. not loaded with DNA but subjected to the normal loading and hybridisation procedure.)

To estimate the background, the signal (a) given by 10ng of argE fragment (arbitrary gene dosage of one) was subtracted from the counts binding (c) to 2 μg of E.coli DNA (arbitrary gene dosage of one). The difference (b) was then the nonspecific background. The line was fitted by linear regression. The value of the background as a fraction of total ($^b/_c$) is 0.44.

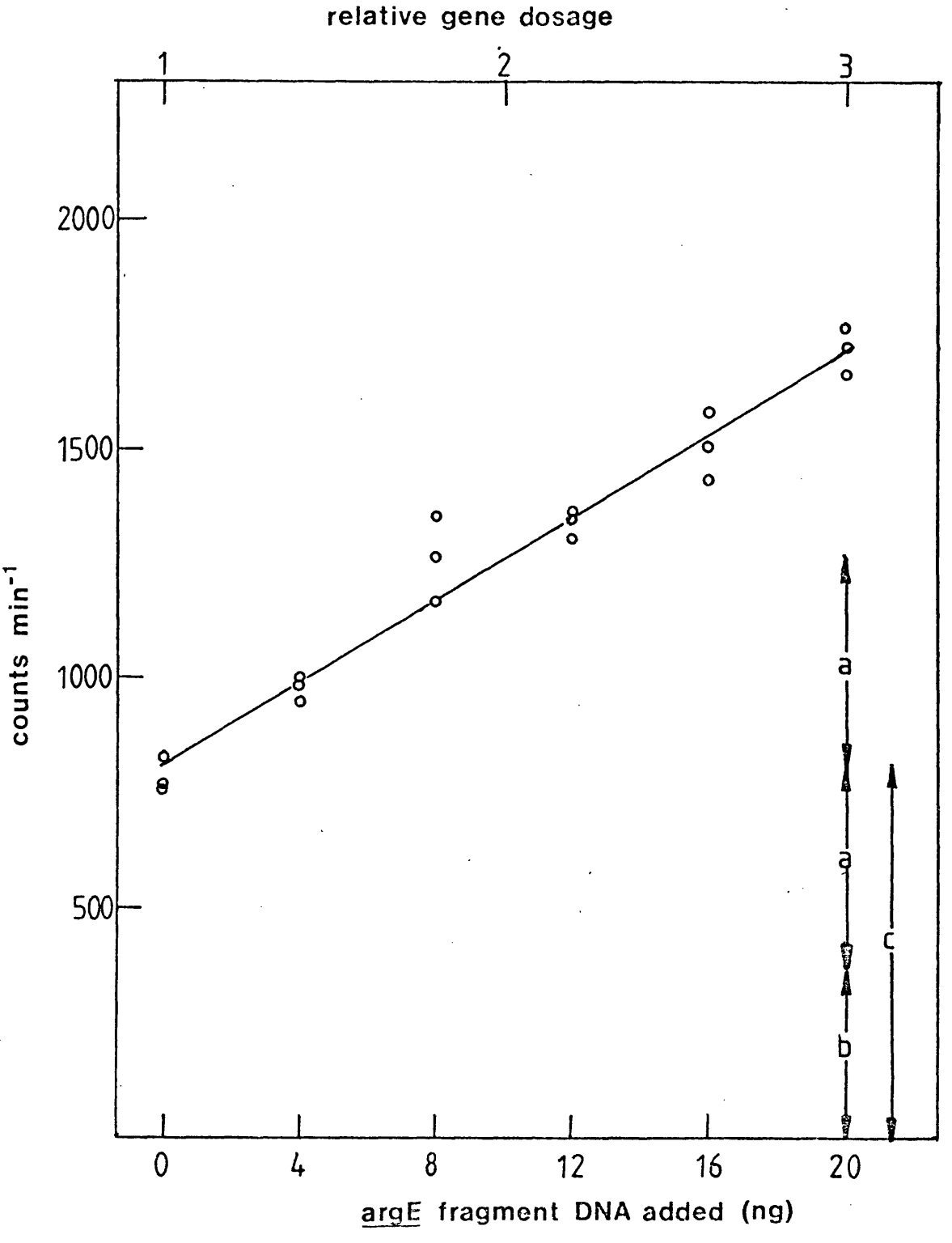
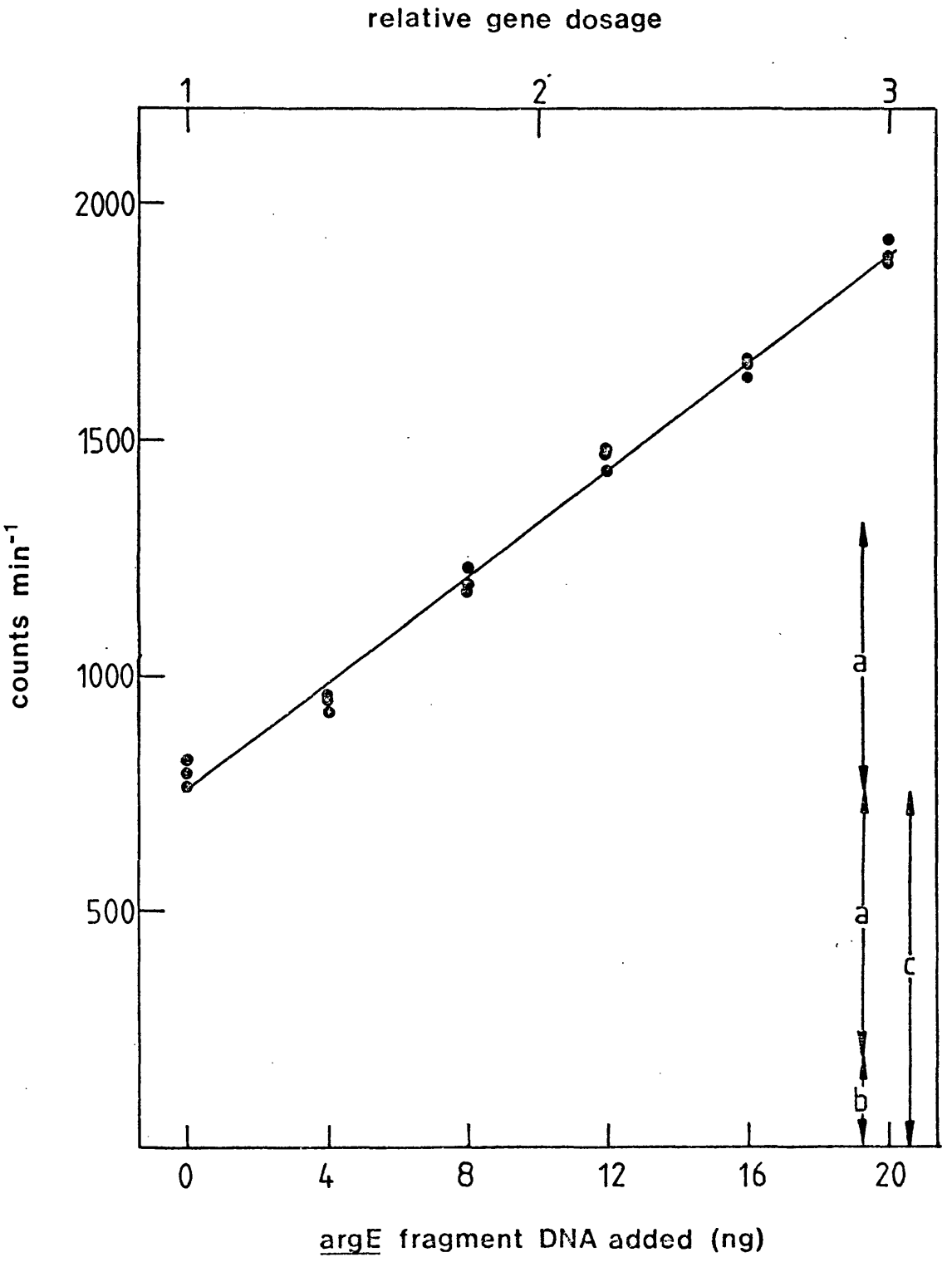

relative gene dosage
1
2
3
2000
1500
1000
500
counts min-1
a
a
b
c
0
4
8
12
16
20
argE fragment DNA added (ng)

Figure 3.7. Second standard curve hybridisation with pLG4 probe

The procedure was as described in the legend to Fig. 3.6 except that the post-hybridisation washes were at 30°C. The experiment was performed in duplicate, unlabelled, salmon DNA (4 $\mu g ml^{-1}$) being added to the probe DNA in one case. Closed circles represent points without salmon DNA, open circles with salmon DNA.
The values for the background as a fraction of the total counts $(\frac{b}{c})$ are: with salmon DNA, 0.23, without salmon DNA, 0.25.


relative gene dosage
1
2
3
2000
1500
1000
500
counts min-1
a
a
b
c
0
4
8
12
16
20
argE fragment DNA added (ng)

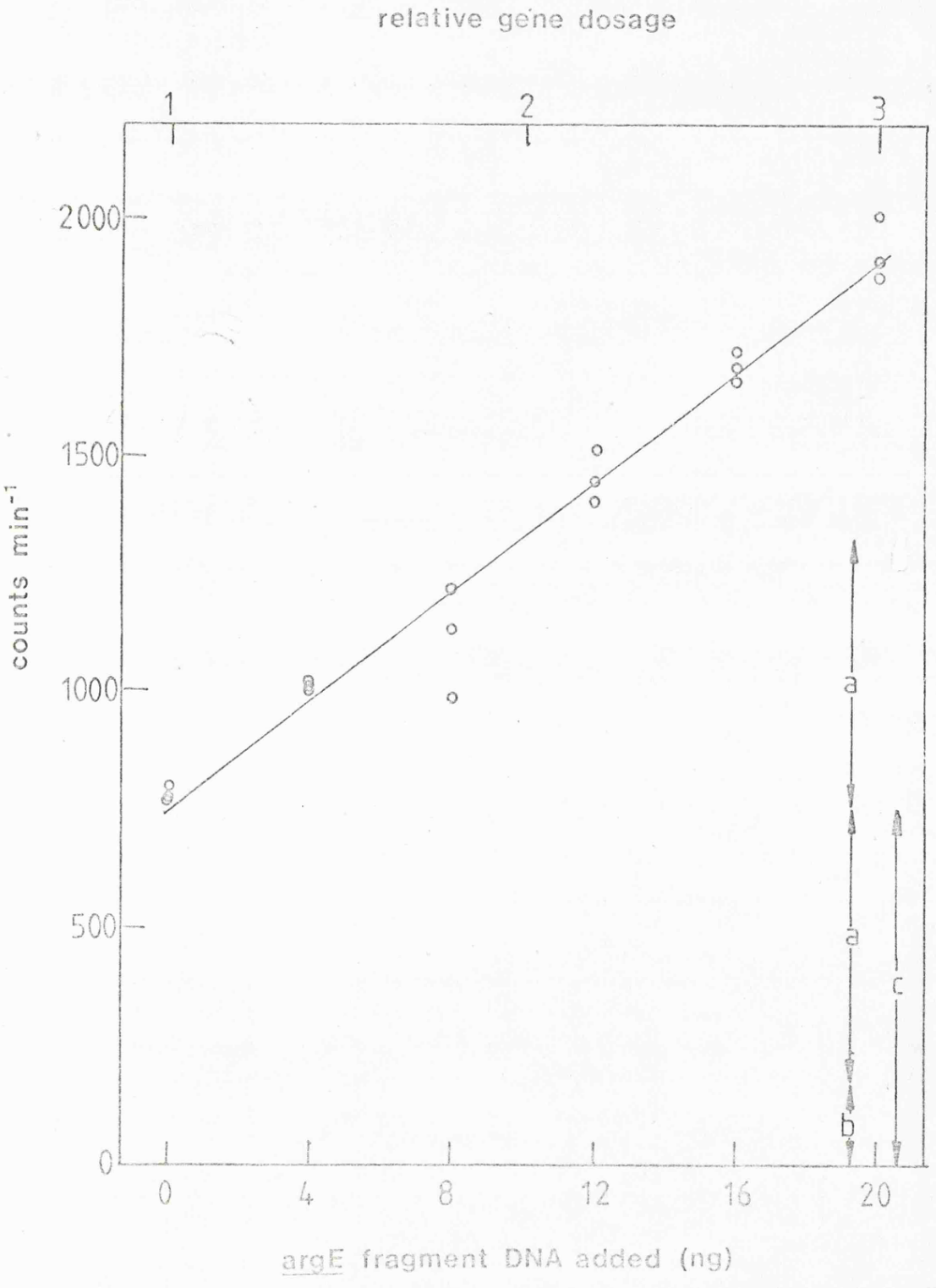
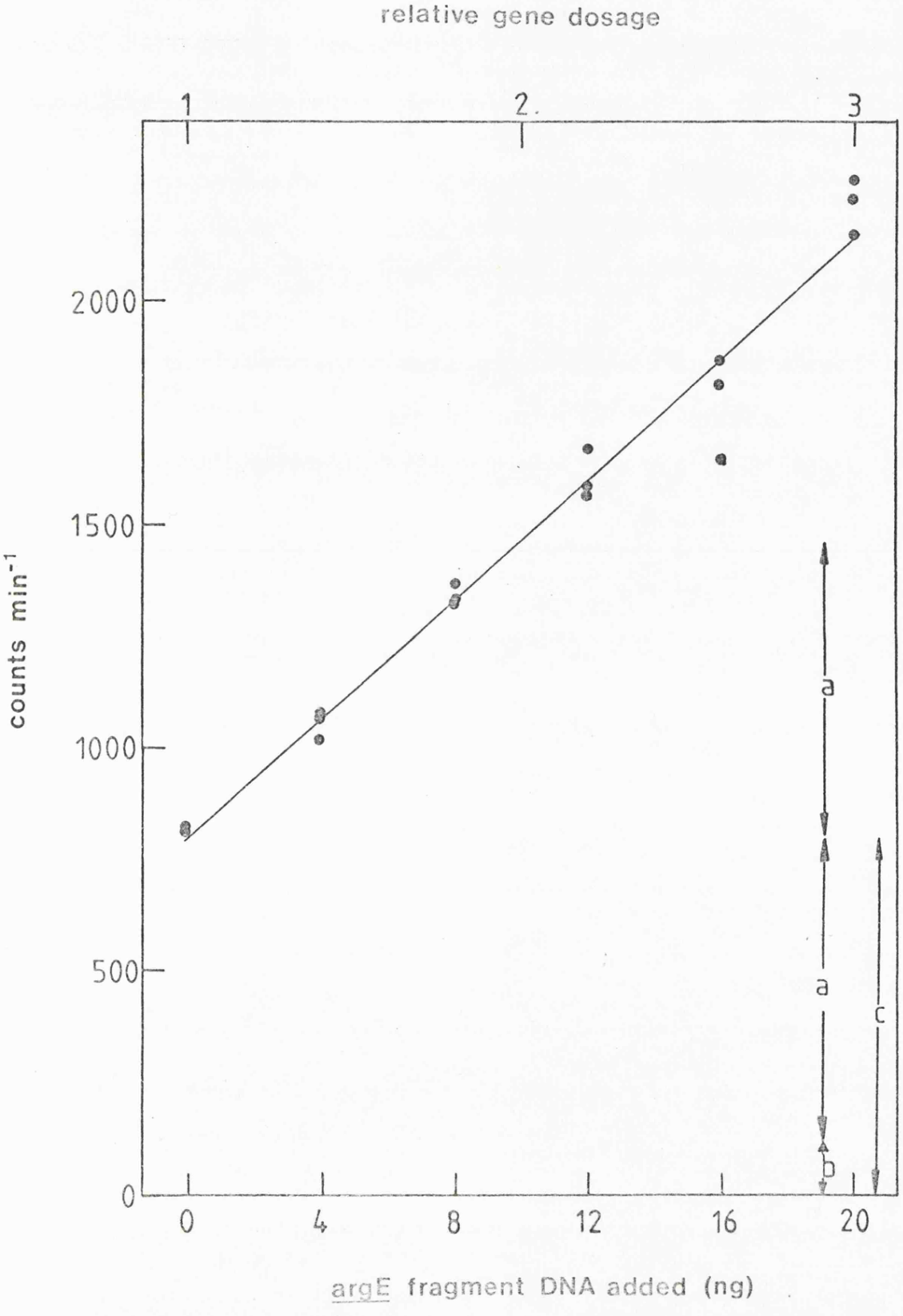
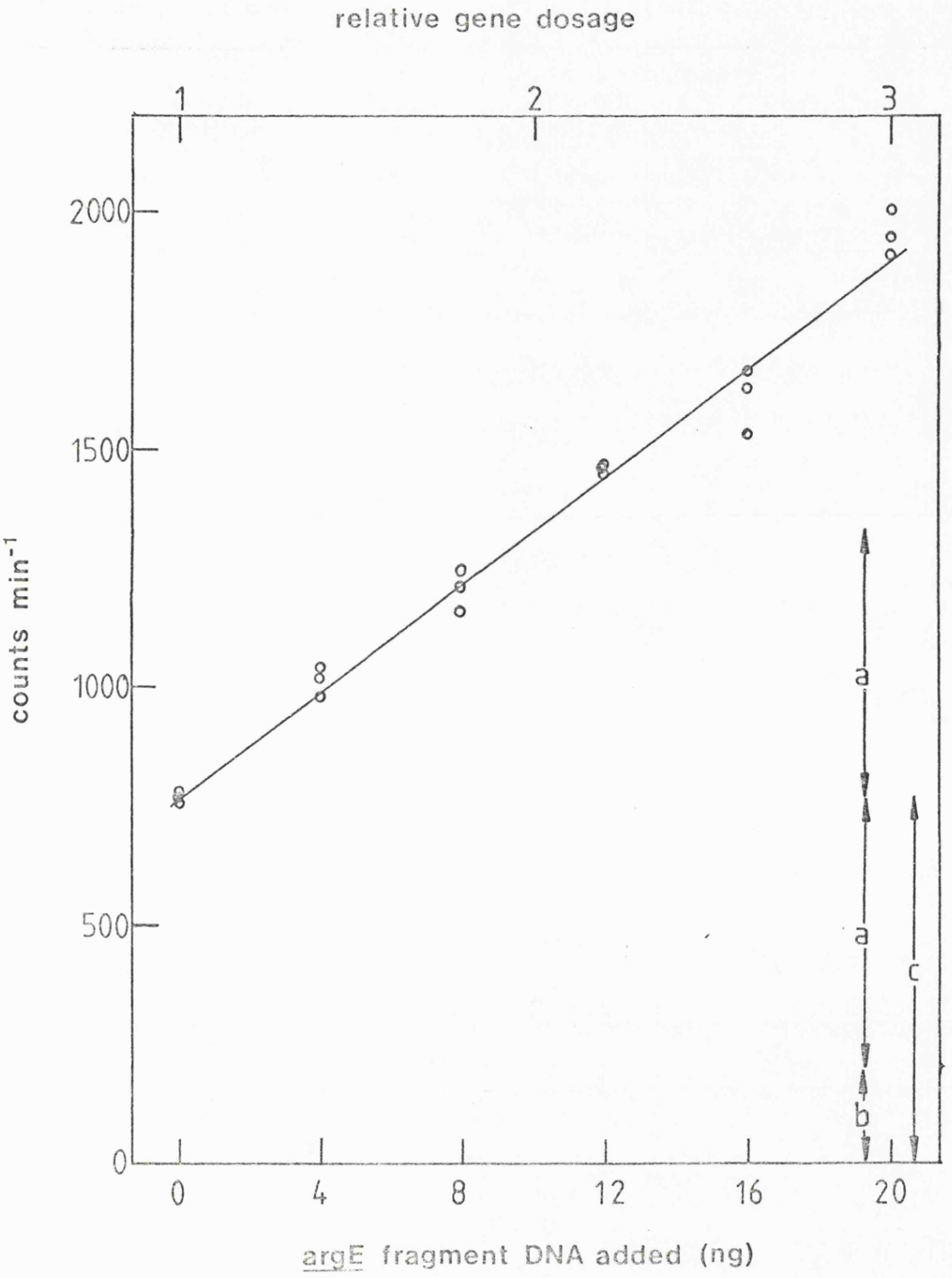

relative gene dosage
1
2
3
2000
1500
1000
500
0
counts min⁻¹
a
a
b
c
0
4
8
12
16
20
argE fragment DNA added (ng)

Figure 3.8. Third standard curve hybridisation with pLG4 probe

The procedure was as described in the legend to Fig. 3.6 except that the hybridisation was at 45°C and the post-hybridisation washes were at 40°C. Successive washes were 30 min in 2xSSC, 50% formamide, 20 min in 2xSSC, 50% formamide and three 20 min washes in 2xSSC. The experiment was performed in duplicate, unlabelled pLG5 DNA (4 $\mu g ml^{-1}$) being added to the probe DNA in one case. Closed circles represent filters hybridised to probe without pLG5 DNA, open circles those with pLG5 DNA.

The values for the background as a fraction of the total counts ($\frac{b}{c}$) are: with pLG5 DNA, 0.25, without pLG5 DNA, 0.15.


relative gene dosage
1
2
3
2000
1500
1000
500
0
counts min⁻¹
0
4
8
12
16
20
argE fragment DNA added (ng)
a
a
b
c

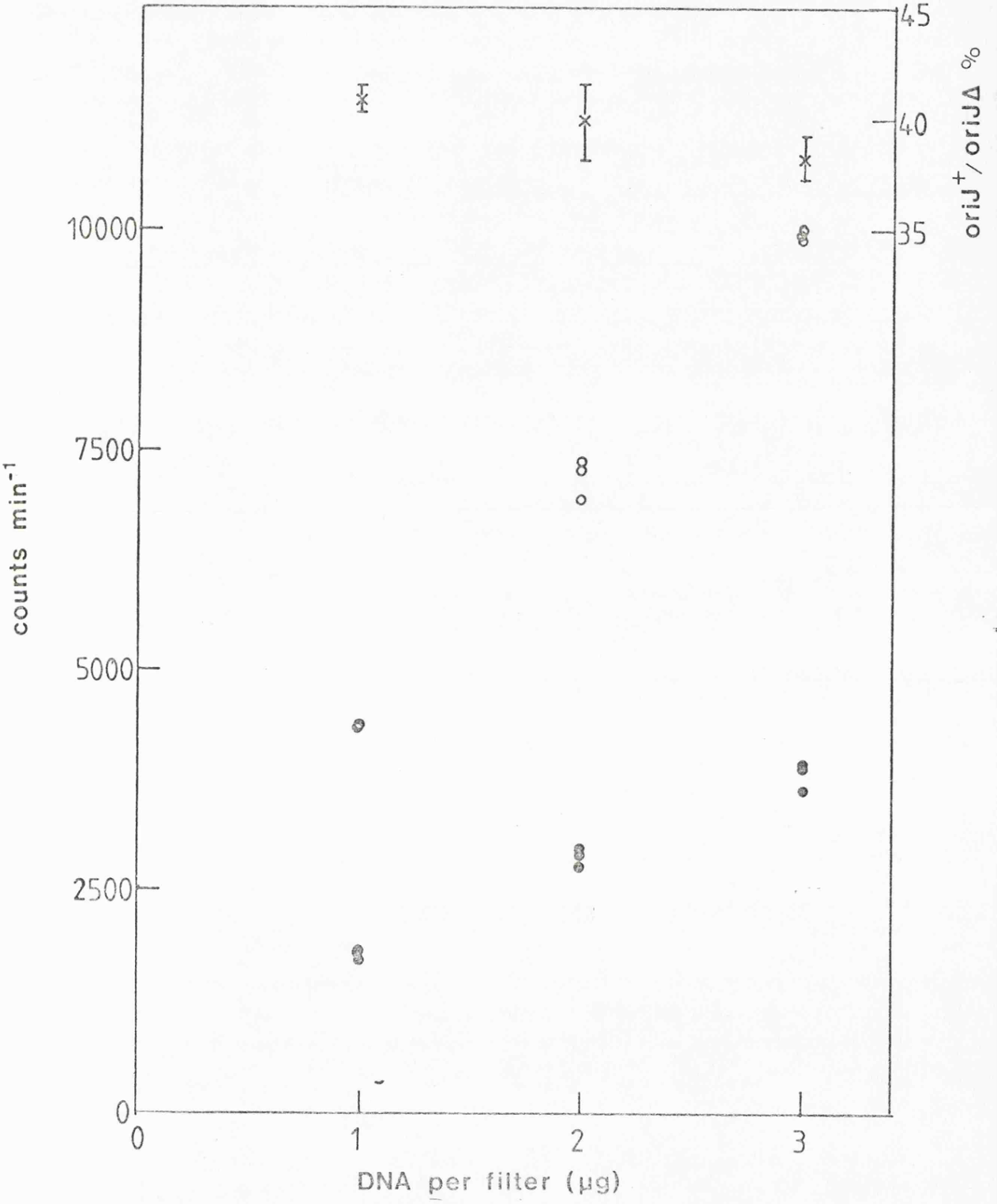

relative gene dosage
1
2
3
2000
1500
1000
500
0
counts min⁻¹
0
4
8
12
16
20
argE fragment DNA added (ng)
a
a
b
c

$\sim$ 40% of the total signal to $\sim$ 25% of the total signal seems to be real.

It was not possible to estimate the background for every probe by such a laborious and indirect method, but a more direct estimation is possible for the probe pLG2 (*oriJ*$^+$) which has no sequence homology with pLG5 as far as is known. The strain AB1157 and its derivatives are known to carry a deletion of the region of the chromosome homologous with pLG2 (see Diaz *et al.*, 1979) thus it was possible by comparing the hybridisation of pLG2 to C600 DNA and AB2463 DNA to estimate the non-specific fraction. This probe even under the more stringent conditions adopted following the experiment described above gave a figure of 40% of the total signal being nonspecific (Fig. 3.9). It is not clear why this probe should give a higher background than pLG4 (*argE*$^+$) though two contributory factors may be suggested. It is known that there is some slight residual homology between AB2463 and pLG2 (Diaz *et al.*, 1979) so that the background as measured in the case of pLG4 might not all be truly non-specific. The extent of the homology is very slight however so that this would be a very minor factor. Secondly the chromosomal fragment in pLG2 (*oriJ*$^+$) is smaller than that in pLG4 (*argE*$^+$), being 8.5Md rather than 12Md, so that a reduced signal and decreased signal : noise ratio is expected assuming that the nonspecific background is dependent upon the total amount of chromosomal DNA loaded onto the filter, whereas the true signal would depend upon the size and number of the sequences in the chromosomal DNA that were homologous with the probe. It is interesting to note that the signal : noise ratio as measured in this experiment is constant over a range of amounts of DNA added to the filter.

Figure 3.9. Direct estimation of nonspecific background

Filters were loaded with the indicated quantities of DNA extracted from stationary phase cultures of C600 oriJ$^+$ (o) or AB2463 oriJΔ (●) and hybridised to pLG2 probe as described in section 2.II ("Kourilsky hybridisation"). The counts binding to the oriJ$^+$ filters are expressed as a percentage of those binding to the oriJΔ filters (×). All counts were corrected by subtracting the mean of the number of counts binding to three blank filters prepared as described in section 2.II.


45
40
35
oriJ+/oriJΔ %
10000
7500
5000
2500
0
counts min-1
0
1
2
3
DNA per filter (µg)

This implies that variations in the amount of DNA added to a filter will not interfere with experimental measurements of marker ratios.

The values of the nonspecific binding of probe DNA found in these experiments make absolute quantitative measurements of marker ratios impractical since the level of background cannot be measured for each probe in each experiment. The differences in marker gradients between two strains or one strain under different conditions may still be studied however. The effect of a high background in such a case would be to reduce the apparent differences so that any attempt to quantify the change will result in an underestimation but the qualitative interpretation will be unaffected. All studies reported here were therefore designed in a comparative manner.

3.IV. "SOUTHERN BLOT" HYBRIDISATIONS

The method of transferring DNA from agarose gels to nitrocellulose filters as developed by Southern (1975) was tested as a possible means of eliminating nonspecific background hybridisation. The method has been widely used to estimate the size of those restriction fragments homologous to a given probe with a view to physical mapping, but though highly repeated fragments may be distinguished from unique ones by their signal strength the technique has not been used to accurately quantify relative gene dosages [a]. If it could be so employed, measurements of relative gene dosages could be internally controlled by the use of several probes in one hybridisation mixture and several different samples of DNA on one filter. It should also prove possible to separate bands of DNA showing specific hybridisation from the mass of other DNA sequences exhibiting only nonspecific background, so that most of the "nonspecific hybridisation" would be eliminated.

In one experiment, increasing amounts of EcoR1 digested pLG4 DNA were loaded into different slots of an agarose gel. Several other slots were loaded with different amounts of EcoR1 digested chromosomal DNA from a stationary E.coli C600 culture. The gel was run overnight, the DNA transferred to a nitrocellulose filter and hybridised with nick translated pLG4 DNA in the absence of dextran sulphate as described (see section 2.II). The radioactive bands on the filter were located by auto-

a. The term 'relative gene dosage' has been defined (Chandler and Pritchard, 1975) as the number of copies of a gene expressed in relation to that of all genes. The term therefore effectively refers to gene copies per unit of DNA.

radiography (Fig. 3.10), cut out and counted in a scintillation spectrometer. As maybe seen from Figs. 3.10 and 3.11, the number of counts binding to the filter was not strictly proportional to the amount of DNA on the filter. In addition, the chromosomal DNA did not give the same intensity of signal as the plasmid DNA band corresponding to the same gene concentration. This might have been due either to some effect of the concentration of DNA in the gel altering the efficiency of transfer to the filter, or to the concentration of DNA on the filter affecting the hybridisation efficiency. To test this, the experiment was repeated using a constant quantity of chromosomal DNA in each slot in addition to the varying amounts of plasmid DNA. Although the strength of the hybridisation signal was more proportional to the amount of argE plasmid DNA on the filter, the chromosomal DNA was still giving a relatively lower signal than the pLG4 DNA (Fig. 3.12). The poor signal given by the chromosomal DNA could be explained by shearing of the DNA prior to digestion. This would lead to smearing of the arg sequences down the gel as is indeed seen in Fig. 3.10. The non-linear response of the signal to the input plasmid DNA may well have been due to the very poor hybridisation conditions in which the reaction only goes 1% to completion at best (A. Jeffreys personal communication).

To overcome the problem of DNA breakage high molecular weight DNA was isolated from strain KL1 by the lysis III method as described (section 2.II) and digested with EcoR1 nuclease under conditions previously shown to digest the DNA just to completion in order to minimise any cleavage by nonspecific nucleases. Hybridisation was

Figure 3.10. A standard curve for hybridisation by the method of Southern (1975)

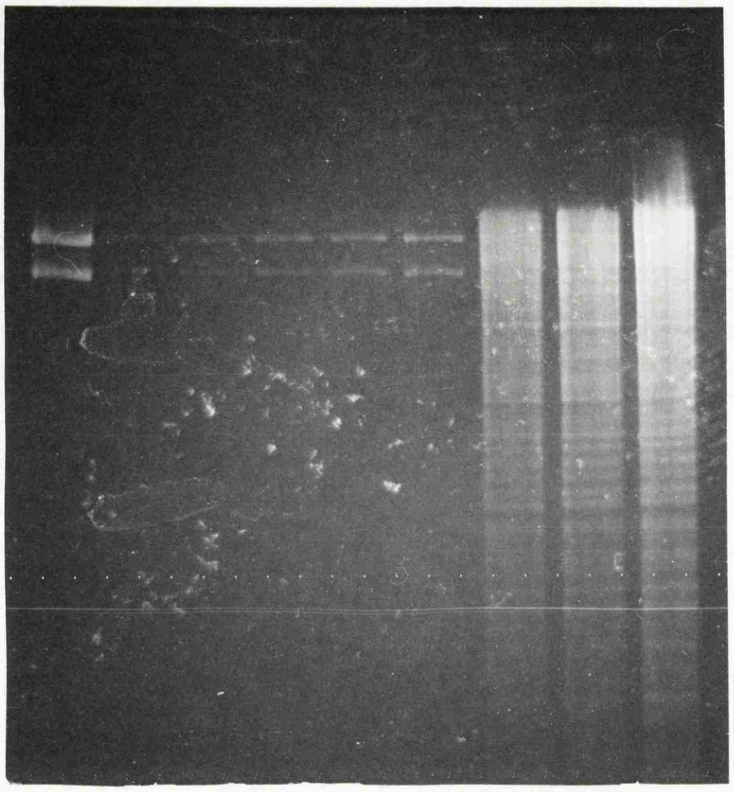
Samples of pLG4 DNA and E.coli C600 DNA were digested with endonuclease EcoR1 and loaded onto an 0.7% agarose gel. The samples were electrophoresed for 11hr at 40v in tris-borate buffer. The procedure for the transfer of the DNA to nitrocellulose, hybridisation to pLG4 probe, autoradiography and scintillation counting is described in section 2.II. The gel slots were loaded with DNA as follows: (a) 100ng pLG4, (b) 1.6ng pLG4, (c) 3.2ng pLG4, (d) 4.8ng pLG4, (e) 6.4ng pLG4, (f) 8.0ng pLG4, (g) 0.25 μg E.coli C600, (h) 0.5 μg E.coli C600, (i) 1.0 μg E.coli C600.

Given molecular weights of 2.5×10^9 for the E.coli chromosome (Cooper and Helmstetter, 1968), 7.3×10^6 for RSF2124 (So et al., 1975) and 1.28×10^7 for the argE EcoR1 fragment of E.coli (Kozlov et al., 1977) then 1 μg of E.coli C600 DNA and 8ng of pLG4 DNA each contain 5ng of argE EcoR1 fragment.

A photograph of gel stained with ethidium bromide

B autoradiograph of filter after hybridisation

A



a b c d e f g h i

B

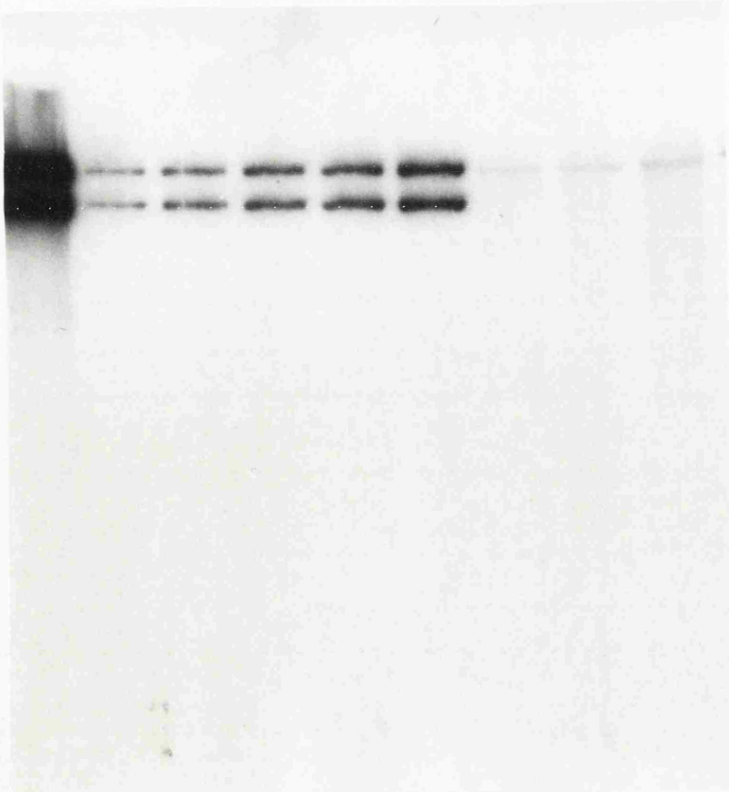


Figure 3.11. A standard curve for hybridisation by the method of Southern (1975)

This figure shows scintillation counts of sections of the nitrocellulose filter shown in Fig. 3.10. Excised pieces of filter corresponding to the 12.8 Md bands of pLG4 (o) or C600 (●). DNA were counted and the counts corrected by substracting the counts obtained from an area of the filter causing no darkening of the autoradiograph.

The scale on the abscissa refers to ng of the E.coli argE EcoR1 fragment DNA calculated from the equivalence given in the legend to Fig. 3.10.

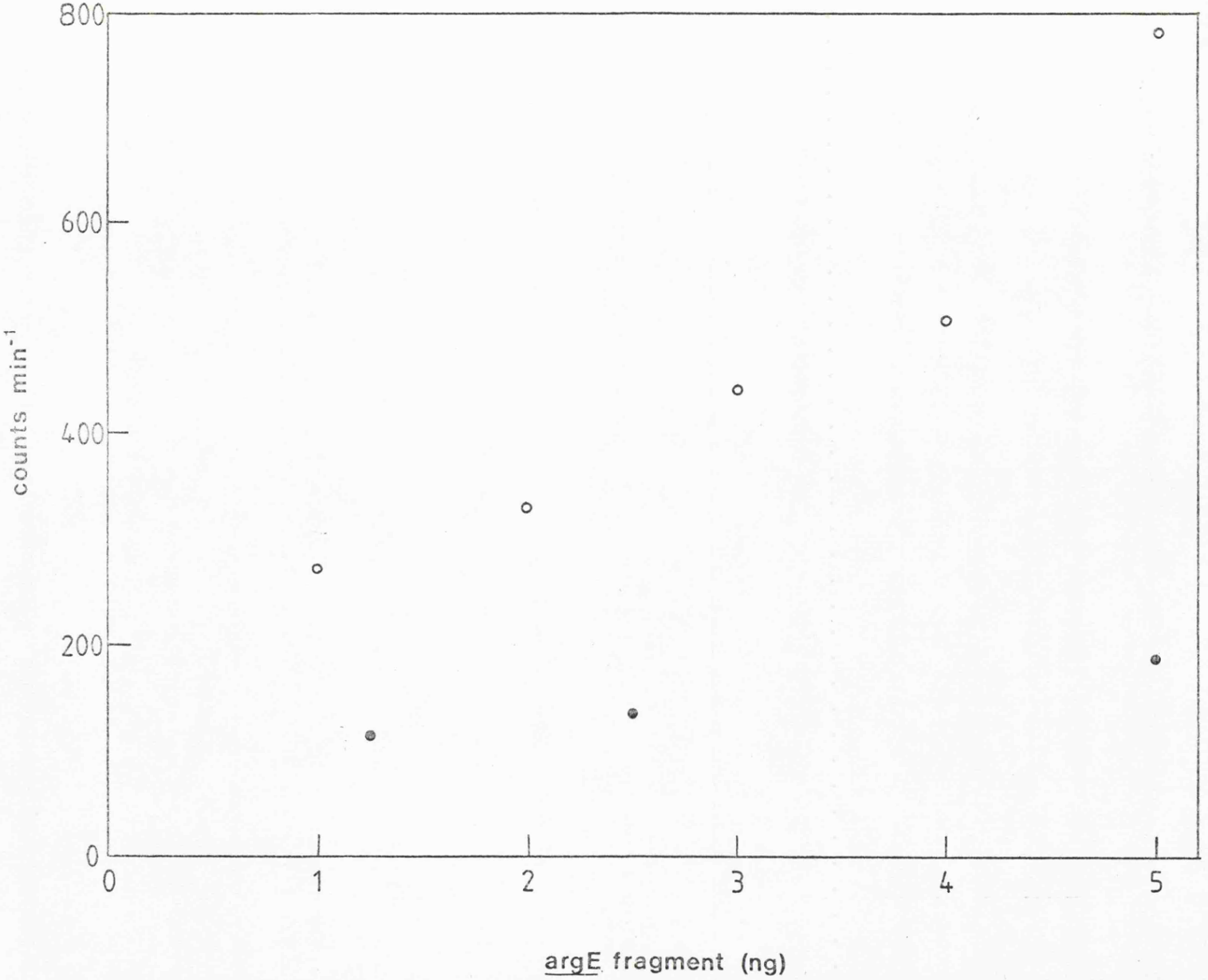
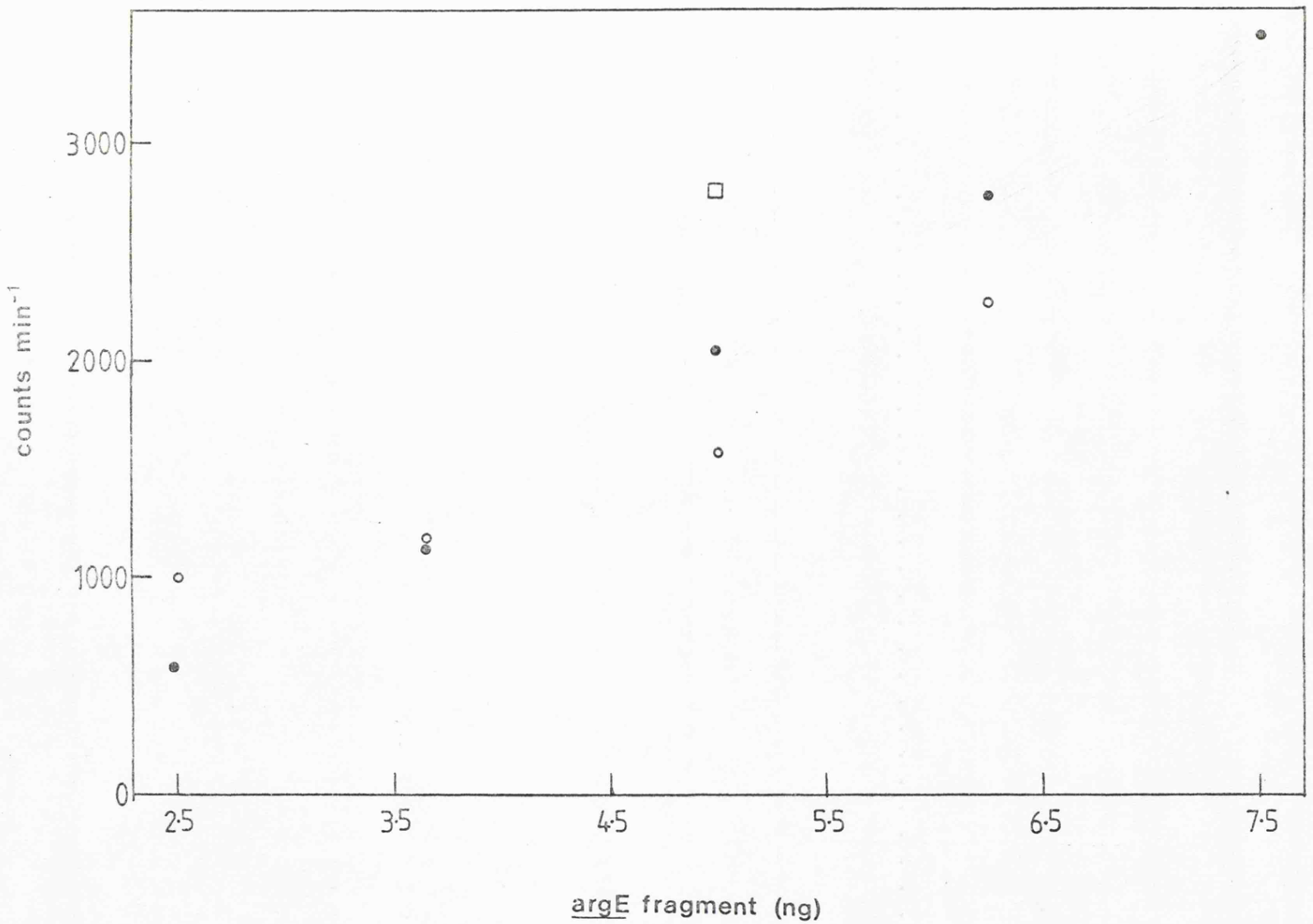

800
600
400
200
0
counts min-1
0
1
2
3
4
5
argE fragment (ng)

Figure 3.12. A second standard curve for hybridisation by the method of Southern (1975)

Samples of pLG4 DNA, E.coli C600 DNA and E.coli W3110 DNA were digested with EcoR1 endonuclease, loaded onto an 0.3% agarose gel, and electrophoresed at 40v for 15hr in tris-borate buffer. The procedures for the transfer of the DNA to nitrocellulose, hybridisation to pLG4 probe, autoradiography and counting of the bands is described in section 2.II. Each slot was loaded with 0.5 µg of C600 DNA plus varying amounts (0-8ng) of pLG4 DNA (o), or with 0.5 µg of W3110 DNA plus varying amounts (0-8ng) of pLG4 DNA (●), or with 8ng of pLG4 DNA only (□).

The figure shows scintillation counts of sections of nitrocellulose filter corresponding to 12.8 Md bands of the autoradiograph (not shown). The counts were corrected by subtracting those obtained from an area of the filter causing no darkening of the autoradiograph.

The scale on the abscissa refers to ng of the E.coli argE EcoR1 fragment DNA calculated from the equivalence given in the legend to Fig. 3.10.


counts min⁻¹
3000
2000
1000
0
2·5
3·5
4·5
5·5
6·5
7·5
argE fragment (ng)

carried out using dextran sulphate in the reaction mixture (see Wahl et al., 1979) in order to increase the hybridisation efficiency upto two orders of magnitude (A. Jeffries personal communication). In this final experiment, EcoR1 nuclease treated KL1 DNA from a rifampicin treated culture was loaded into 5 slots of a gel and EcoR1 nuclease treated KL1 DNA from a culture growing exponentially at a doubling time of 24 min was loaded into 5 other slots. After running and blotting of the chromosomal samples, a mixture of equal amounts of pLG4, pLG5, pLG18 and pLG19 DNA was used as probe for hybridisation. The bands were cut out and counted as described (section 2.II). The results are shown in Fig. 3.13 and Table 3.2. The measured differences in relative gene dosage between the two cultures were much larger than those calculated from the growth rates of the cultures. This again is probably due to under recording of the less intense bands.

It must be concluded that this method, although promising from a theoretical standpoint is not technically suited for the quantitative estimation of gene dosage.

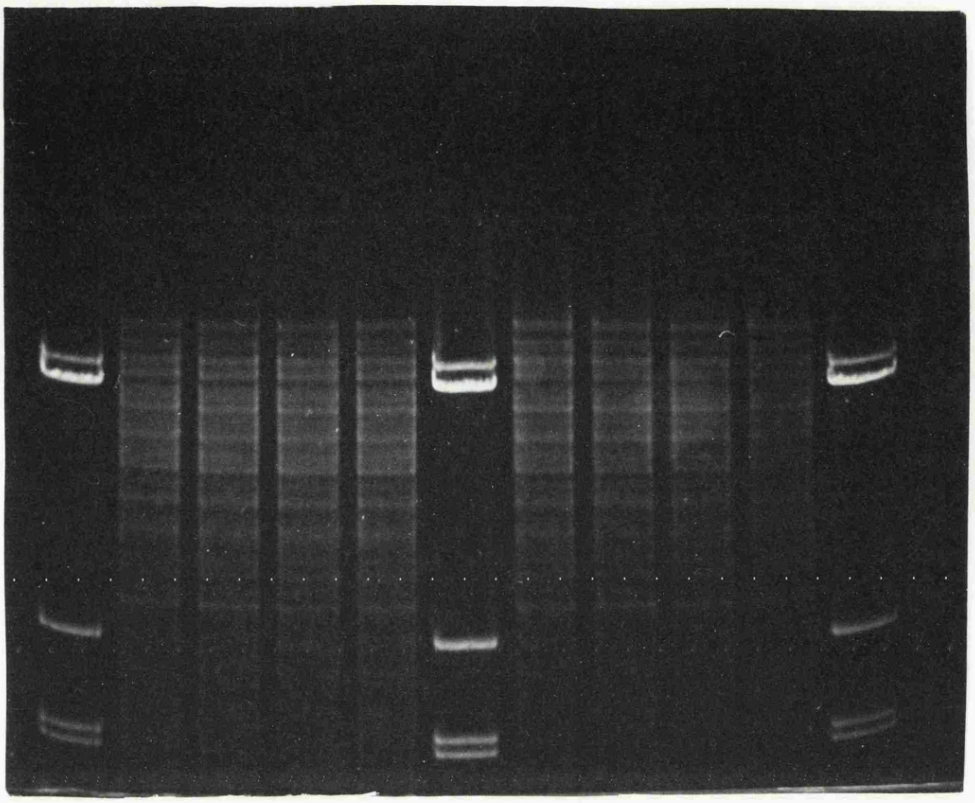
Figure 3.13. Gene dosage analysis by "Southern" hybridisation

E.coli KL1 DNA was isolated from an exponentially growing broth culture and from a culture treated with rifampicin to block initiation of chromosome replication and produce only completely replicated chromosomes (see section 4.I). The DNA was digested with a carefully predetermined quantity of endonuclease EcoR1 in order to just digest to completion. Samples were loaded onto an 0.5% agarose gel and run for 17 hr at 40v in tris-borate buffer. The tracks were loaded as follows: (b-e) "exponential" KL1 DNA, 0.2 µg per slot, (g-j) "aligned" KL1 DNA, 0.2 µg per slot, (a, f and k) λ DNA (EcoR1 digested, 0.5 µg per slot). The procedure for the transfer of the DNA to nitrocellulose, hybridisation to pLG4 probe, autoradiography and scintillation counting is described in section 2.II. The probe was a mixture (75ng each) of pLG4 (argE), pLG5 (proA), pLG18 (manA) and pLG19 (purF) nick translated in the same reaction mixture.

A. Photograph of the gel stained with ethidium bromide.

B. Autoradiograph of the filter after hybridisation.

A



a b c d e f g h i j k

B

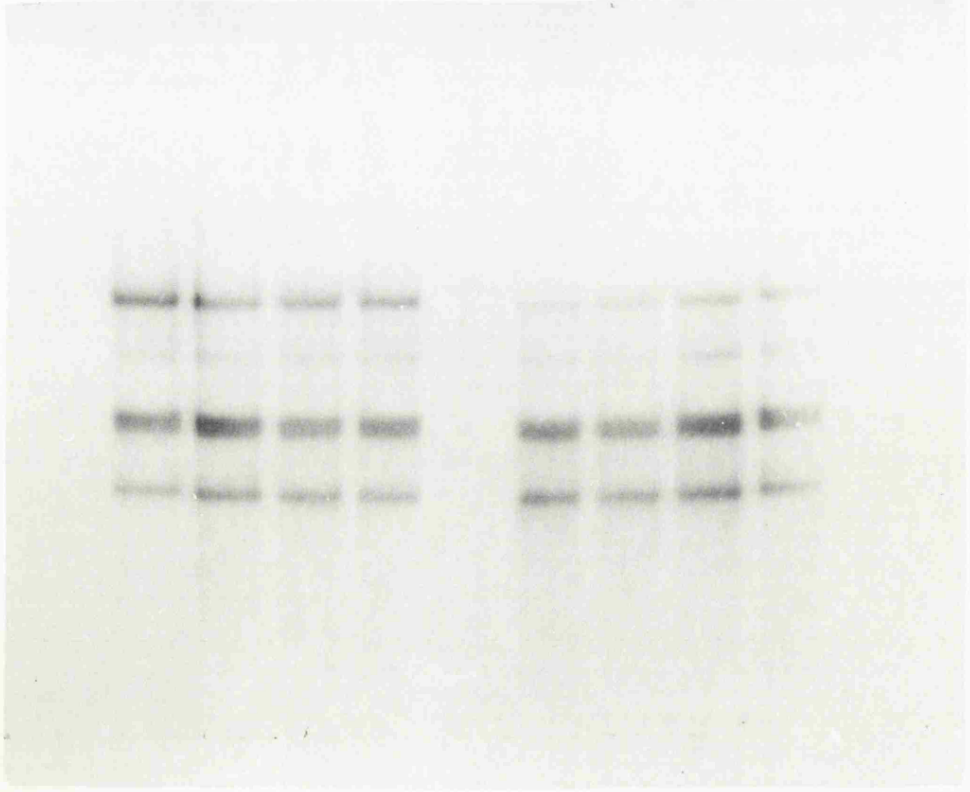


Table 3.2a. Gene dosage analysis by "Southern" hybridisation

Scintillation counts of bands excised from the filter shown in Fig. 3.13. Blanks were taken from below the purF band and counting was done as explained in section 2.II and Fig. 3.11 legend.

marker	slot b	slot c	slot d	slot e	mean a-e corrected for blank
argE	233.36	204.56	168.00	186.24	125.42
proA	138.48	154.72	149.36	128.24	70.08
manA	233.20	270.56	204.96	219.52	159.44
purF	165.04	188.08	163.92	143.76	92.58
blank	67.04	88.00	71.68	63.76	-

marker	slot g	slot h	slot i	slot j	mean g-j corrected for blank
argE	106.40	122.16	141.68	118.00	30.92
proA	110.80	106.96	133.76	102.88	22.45
manA	223.92	207.20	277.20	214.56	139.58
purF	176.08	181.88	219.44	161.04	93.46
blank	95.34	92.80	96.00	80.48	-

Table 3.2b. Gene dosage analysis by "Southern" hybridisation

The table shows the results of scintillation counting of bands shown in Fig. 3.13. Counting was done as explained for Fig. 3.11. Blanks were cut from just below the purF band.

marker	growing stationary	growing stationary standardised against argE	theoretical marker ratios[a] standardised against arg	Results[b] from section 4.I
argE	4.056	1.000	1.000	1.000
proA	3.121	0.769	0.682	0.679
purF	0.990	0.244	0.515	0.543
manA	1.142	0.281	0.369	0.457

a. Theoretical marker ratios were calculated as follows:

$$\frac{F}{M} = \frac{2^{-Cx/\tau}}{K} \quad \text{..... (Chandler and Pritchard, 1975)}$$

gives $$\frac{\bar{F}_1}{\bar{F}_2} = 2 \exp C(x_2 - x_1)/\tau$$

where F is the mean dosage of a given gene, C is the replication time of the chromosome, τ is the generation time of the culture and x_1 is the fraction of the replication time after initiation at which gene F_1 replicates. τ for the culture used was 23 min (section 4.I), C was taken to be 40 min and values of x were calculated from map positions given by Bachmann and Low (1980)

b. Gives results from the experimental data for strain KL1 used to produce tables 4.1 and 4.2. The values are corrected for blanks and standardised against argE.

CHAPTER 4

4.I. INVESTIGATION OF THE PATTERN OF REPLICATION IN Hfr STRAINS

As outlined in section 1.IX, one aim of this study was to investigate whether the integrated F plasmid influences the pattern of chromosome replication in steady state cultures of E.coli. Accordingly the patterns of replication in two Hfr strains and their isogenic F^- counterparts were investigated. KL99 and AB313 are fairly stable Hfr strains and both are known to give rise to F' plasmids (Low, 1972) implying that the integrated F plasmids have active replication functions. A culture of KL99 was plated out for single colonies and the colonies were tested for transfer of trp his xyl proA and galK by the method of Low (1973) using BB20 and BB21 as recipient strains. Most of the clones gave the expected gradient of markers with the rec^+ recipient and only occasional colonies with the recA recipient. One such clone was scraped off the original master plate into glycerol, frozen at -80°C and used to inoculate cultures for future experiments. Both KL99 and KL1 (F^-) were tested for male specific phage MS2 sensitivity. KL99 was sensitive and KL1 was resistant as expected. Cultures of KL1 and KL99 were grown in exponential phase in 200ml of L broth at 37°. At A_{450} 0.2, 100ml samples were taken for DNA purification by "lysis III" as described in section 2.II. 100ml of fresh pre-warmed broth was then added to the cultures, which were grown for a further generation before a second sample was taken, and the process repeated four times. Six independent samples from each strain were obtained in this way and designated K1-K6 (KL99) and L1-L6 (KL1). The taking of

independent samples after successive dilutions and regrowth of the culture was to ensure that the cultures were truly in steady state conditions and to control for slight fluctuations in growth rate. Both strains grew with a doubling time of 23 $\pm$ 2 min throughout the procedure. 400ml cultures of these two strains were also treated with rifampicin (150 μgml^{-1}) in order to block initiation and produce a constant gene dosage for all genes. 100ml samples were taken after 60, 90 and 120 min and designated M1-M3 (KL99) and N1-N3 (KL1). These samples of DNA were analysed for differences in relative gene dosages by DNA-DNA hybridisation.

In one experiment, each of three samples of DNA from each strain under each growth condition was hybridised against each of three labelled probe DNA samples. The probes used were pLG4 to assay the level of genes in the argE region, pLG5 to assay genes in the proA region and pVH5 to assay genes in the trp region, near to the point of insertion of F in the KL99 chromosome. For each sample of DNA, the number of counts binding to the loaded filter was expressed as a fraction of those binding using pLG4 (argE$^+$) in order to correct for small differences in concentration between batches of DNA as loaded onto the filters. The means of each ratio for KL1 DNA and KL99 DNA were then compared. For ease of comparison these are expressed as a further ratio KL99/KL1. The significance of the differences of these probes X/pLG4 values was tested statistically using the t test. The results are shown in Table 4.1. The values for the exponentially growing cultures did not differ significantly for the proA region of the chromosome, the values for the argE region being equal by definition. A ratio of 1.165 for the Hfr/F$^-$ values was seen for the

Table 4.1. Ratios of counts obtained using different hybridisation probes with KL1 and KL99 DNA : Experiment 1

DNA from exponentially growing cultures

KL99DNA sample	$\frac{trp}{argE}$ (Hfr)	KL1DNA sample	$\frac{trp}{argE}$ (F^-)
K2	0.7528	L2	0.5857
K4	0.7599	L4	0.6644
K6	0.7766	L6	0.7147
mean (x)	0.7631	mean (x)	0.6549

$\frac{x \text{ Hfr } (trp)}{x \text{ } F^-} = 1.165$

t = 2.833 significant at 95% level

KL99DNA sample	$\frac{proA}{argE}$ (Hfr)	KL1DNA sample	$\frac{proA}{argE}$ (F^-)
K2	1.1988	L2	1.1996
K4	1.2636	L4	1.2531
K6	1.3152	L6	1.2335
mean (x)	1.2592	mean (x)	1.2288

$\frac{x \text{ Hfr } (proA)}{x \text{ } F^-} = 1.025$

t = 0.797 not significant at 95% level

Table 4.1. Continued.

DNA from rifampicin treated cultures

KL99DNA sample	trp (Hfr) argE	KL1DNA sample	trp (F^-) argE
M1	1.2038	N1	0.8720
M2	1.0243	N2	1.1114
M3	1.1606	N3	1.2045
mean (x)	1.1296	mean (x)	1.0626

$\frac{x \text{ Hfr (trp)}}{x \ F^-} = 1.063$

t = 0.593 not significant at 95% level

KL99DNA sample	proA (Hfr) argE	KL1DNA sample	proA (F^-) argE
M1	1.6586	N1	1.7717
M2	1.7772	N2	1.7653
M3	1.9778	N3	1.8861
mean (x)	1.8045	mean (x)	1.8077

$\frac{x \text{ Hfr (proA)}}{x \ F^-} = 0.998$

t =-0.032 not significant at 95% level

The t test is used to test the significance of the difference between x (Hfr) and x (F^-).

trp region of the chromosome. This much larger difference is statistically significant within 95% confidence limits using the t test. As expected the marker ratios for the rifampicin treated control cultures were not significantly different in any case.

The trp region of the chromosome therefore had a higher relative dosage in the Hfr strain than in the F^- strain. One possible explanation of this result would be that the KL99 isolate used contained a high proportion of cells which carried F' trp plasmids. To investigate this possibility an interrupted mating experiment was performed as described (section 2.II) using strain BB20 as the recipient. 161 Trp^+ transconjugants obtained during the first 15 min of mating were tested for sensitivity to 'phage MS2 as a test for the presence of the F plasmid using strains KL99 (Hfr) KL20 (F^+), LE500 (F'lac), KL1 (F^-) and W3110 (F^-) as controls. All 161 transconjugants were clearly MS2 resistant. The KL99 clone used seems therefore to be substantially free of F' trp plasmids.

The increase in the relative gene dosage of trp found in KL99 over that found in KL1 therefore seems to reflect a real difference in the pattern of chromosomal replication between the two strains. Since the level of proA was not affected, this difference is unlikely to be due to a difference in the rate of replication fork movement. The data fit better with the proposition that there is initiation from the F plasmid origin of replication in KL99. If this were the true situation all of the markers in the terminal part of the chromosome on the same replication arm should show a raised relative gene dosage in KL99 whilst other markers should not.

As a more rigorous test of this prediction a repeat hybridisation experiment was performed. All twelve of the DNA samples isolated from the growing cultures were used with the six from the aligned cultures. The probes used were again pLG4 (*argE*) and pVH5 (*trp*), plus pLG2 (*oriJ*), pLG18 (*manA*) and pLG19 (*purF*). The results, processed as before, are shown in Table 4.2. The observed *trp*/*arg* and *oriJ*/*arg* ratios were both significantly raised in KL99 relative to those in KL1, the t test giving confidence limits of 95% and 99.9% respectively. None of the other marker ratios were significantly different in the two strains when growing exponentially. These results are consistent with the proposition that there is initiation at the F origin. The position of the *E.coli* replication terminus is not precisely known but a recent report places it between *manA* and *rac* (Louarn *et al.*, 1979). As *oriJ* and *rac* are located on the same cryptic prophage (Diaz *et al.*, 1979) it seems reasonable to suppose that the terminus is located between *oriJ* and *manA*. Thus the two markers whose levels were found to be raised in KL99, *trp* and *oriJ* are on the same arm of the chromosome as F while *manA* and *purF* whose levels were not significantly raised are probably both on the other arm. The *purF*/*argE* value was in fact lower in KL99 than in KL1 but the difference is not significant at the 95% level by test. If it were significant it would only emphasise the assymmetry between the two arms of the KL99 chromosome.

Again the control cultures showed no significant difference in the experimental values between the two strains with the single exception of *manA*, where a relatively large and statistically significant difference was found between the two strains. One explanation for the change in only this single marker might be that KL99 but not KL1 carries a small

Table 4.2. Ratios of counts obtained using different hybridisation probes with KL1 and KL99 DNA : Experiment 2

DNA from exponentially growing cultures

KL99DNA sample	$\frac{trp}{argE}$ (Hfr)	KL1DNA sample	$\frac{trp}{argE}$ (F^-)
K1	0.7518	L1	0.6857
K2	0.7370	L2	0.7198
K3	0.7372	L3	0.6815
K4	0.8582	L4	0.7219
K5	0.7372	L5	0.7442
K6	0.8324	L6	0.7462
mean (x)	0.7756	mean (x)	0.7165

$\frac{x \text{ Hfr (trp)}}{x \text{ } F^-} = 1.083$

t = 2.353 significant at 95% level

KL99DNA sample	$\frac{oriJ}{argE}$ (Hfr)	KL1DNA sample	$\frac{oriJ}{argE}$ (F^-)
K1	4.7141	L1	3.7665
K2	4.8696	L2	3.7692
K3	4.4849	L3	4.0285
K4	4.8208	L4	3.5060
K5	4.1993	L5	3.4896
K6	4.8423	L6	4.2208
mean (x)	4.6552	mean (x)	3.7968

$\frac{x \text{ Hfr (oriJ)}}{x \text{ } F^-} = 1.226$

t = 5.383 significant at 95% level [a]

Table 4.2. Continued.

KL99DNA sample	manA/argE (Hfr)	KL1DNA sample	manA/argE (F^-)
K1	1.3325	L1	1.1799
K2	1.4180	L2	1.3341
K3	1.4392	L3	1.4212
K4	1.5696	L4	1.3365
K5	1.2984	L5	1.3799
K6	1.3844	L6	1.4296
mean (x)	1.4070	mean (x)	1.3468

x Hfr (manA) = 1.045
x F^-

t = 1.118 not significant at 95% level

KL99DNA sample	purF/argE (Hfr)	KL1DNA sample	purF/argE (F^-)
K1	1.1188	L1	1.2015
K2	1.0981	L2	1.2536
K3	1.1653	L3	1.2833
K4	1.2456	L4	1.1666
K5	1.1480	L5	1.2461
K6	1.2634	L6	1.2690
mean (x)	1.1732	mean (x)	1.2367

x Hfr (purF) = 0.949
x F^-

t = -1.934 not significant at 95% level

Table 4.2. Continued.

DNA from rifampicin treated cultures

KL99DNA sample	trp/argE (Hfr)	KL1DNA sample	trp/argE (F^-)
M1	1.2169	N1	1.0974
M2	1.0620	N2	1.1114
M3	1.1334	N3	1.1925
mean (x)	1.1374	mean (x)	1.1338

$\frac{x \text{ Hfr (trp)}}{x \text{ } F^-} = 1.003$

t = 0.067 not significant at 95% level

KL99DNA sample	oriJ/argE (Hfr)	KL1DNA sample	oriJ/argE (F^-)
M1	8.693	N1	7.429
M2	7.089	N2	7.833
M3	6.734	N3	8.180
mean (x)	7.506	mean (x)	7.814

$\frac{x \text{ Hfr (oriJ)}}{x \text{ } F^-} = 0.961$

t = 0.487 not significant at 95% level

Table 4.2. Continued.

KL99DNA sample	manA/argE (Hfr)	KL1DNA sample	manA/argE (F^-)
M1	2.6145	N1	2.8381
M2	2.4979	N2	2.9468
M3	2.3956	N3	3.0837
mean (x)	2.5027	mean (x)	2.9562

x Hfr (manA) / x F^- = 0.847

t = -4.771 significant at 95% level [b]

KL99DNA sample	purF/argE (Hfr)	KL1DNA sample	purF/argE (F^-)
M1	2.4076	N1	2.2523
M2	2.1083	N2	2.2560
M3	2.1695	N3	2.3195
mean (x)	2.2284	mean (x)	2.2759

x Hfr (purF) / x F^- = 0.979

t = -0.498 not significant at 95% level

The t test is used to determine the statistical significance of the difference between x (Hfr) and x (F^-).

(a) significant at 99.9% level

(b) significant at 98% level

deletion of the chromosome in the region covered by pLG18. This seems unlikely since both strains are directly derived from the same parent strain KL20 F^+ (Bachmann, 1972 and B.J. Bachmann personal communication), but as KL99 has acquired a lac$^-$ mutation during this step (Bachmann, 1972), it is conceivable that it may also have acquired a deletion. If this is indeed the case the observed man/arg ratio seen in the growing KL99 culture would need to be corrected to reflect this. The result would be an increased relative gene dosage for the manA region in KL99 and a KL99 man/KL1 man ratio of $\sim$ 1.15-1.20. Since the gene dosage level of the pur F region is not raised in KL99 relative to KL1 this again could not be explained as a reflection of a difference in the rate of fork movement, but could be explained by saying that manA is so close to terC that there is some replication through terC. Although it is known that termini of replication do slow the progress of replication forks rather than forming a complete block (see section 1.II), the observed difference in the two aligned cultures may not be real and it seems simpler to explain it as a freak result than to invoke such tortuous arguments.

Taken together, the two hybridisation experiments indicate a significant rise in the gene dosage of the terminal part of that arm of the KL99 chromosome into which the F plasmid is integrated. This is all the more remarkable since the presence of the integrated plasmid will increase the length of that arm of the chromosome by 4% - 5% which at such a fast growth rate would be expected to significantly lower the relative gene dosage of those markers to the terminal side of it. The only reasonable explanation of the results seems to be that there was a

"switch on" or more precisely there was no "switch off" of the F initiation control.

What of the converse? Chandler et al. (1976) were unable to demonstrate a convincing level of initiation from the F origin during exponential growth using an Hfr in which F was inserted near lac, closer to oriC than the point of integration of F in KL99, though they did demonstrate such initiation following release of a block imposed by thymine limitation. It would be instructive to carry this a step further and investigate an Hfr strain in which F was integrated very close to oriC or an Hfr growing extremely slowly. As explained in section 1.IX no significant effect on marker ratios is expected in either case, but a large effect on the DNA/mass ratio is expected in the former. Strain AB313 which carries the F plasmid integrated extremely close to oriC (Low, 1972; Bachmann and Low, 1980) was chosen for further study, by the measurement of the DNA/mass ratio.

Twenty single colonies of Hfr AB313 were tested for transfer as described for KL99. The strains AT2092 (rec^+) and LE521 (recA) were used as recipients. All 20 isolates transferred the markers xyl malA pheA purF his and argH in the expected order and all gave few transconjugants with the recA strain (pheA was not tested with LE521). Two isolates were selected for use. Isolate 1 was typical, whereas isolate 15 seemed to give a higher than average level of transconjugants both with AT2092 and with LE521. Each isolate gave a similar result in the subsequent work (see later). These isolates and a purified clone of the isogenic F^- strain AB253 were tested for MS2 sensitivity with the expected result. These three clones were grown in M9 casamino acids medium, and

whilst in exponential phase at A_{450} of 0.25, duplicate samples were taken from each of the three cultures into an equal volume of 15% trichloroacetic acid. The samples were processed and analysed by the diphenylamine procedure as described (section 2.II). The results are shown in Table 4.3. A slightly higher DNA/mass ratio was observed in the Hfr than in the F^- strain. This was partly due to the difference in growth rate of the two strains. When the figures are adjusted to take account of this the difference between the two strains becomes $\sim$ 9%. If the F origin in the Hfr were initiating at the same rate as in the autonomous state a roughly two-fold difference would be expected. As no such effect was observed in this experiment it is probable that the F plasmid is regulating its own copy number rather than its rate of initiation (see discussion).

Table 4.3. The DNA/mass ratios of AB253 and two AB313 isolates

Strain	Diphenylamine readings		Mean A_{450} of culture		DNA/mass	$\frac{\text{Strain x}}{\text{AB253}}$
AB253	0.339	0.358	0.349	0.248	1.407	1.000
AB313-1	0.395	0.420	0.408	0.258	1.581	1.124
AB313-15	0.390	0.422	0.406	0.258	1.574	1.119

Strain	Generation time (τ)	G/M [a]	$\frac{\text{DNA}}{\text{mass}}$ (τ=40) [b]	$\frac{\text{Strain x}}{\text{AB253}}$ corrected for τ
AB253	34	0.948	1.484	1.000
AB313-1	37	0.976	1.620	1.091
AB313-15	37	0.976	1.613	1.087

a Calculated from $\frac{G}{M} = \frac{\tau(1-2^{-C/\tau})}{KC\ln 2}$ Pritchard and Zaritsky (1970)

where G is the mean number of genome equivalents per cell, M is the mean cell mass, τ is the generation time, C is the replication time of the chromosome and K is a constant. A value of 40 min was assumed for C and of $\frac{1\tau}{2\ln 2}$ for K, to make G/M = 1 when τ = 40 min.

b The DNA/mass was normalised to the expected value when τ = 40 by dividing by G/M in order to correct for the difference in growth rate between the two strains.

4.II. CHROMOSOME REPLICATION IN A *dnaA46* STRAIN OF *E.coli*

Several groups of workers have shown that there is a stimulation in the rate of DNA synthesis when a *dnaA* strain growing at a semi-permissive temperature is treated with chloramphenicol (see section 1.X). The stimulation has been thought to be due to extra initiation and in this chapter results will be presented that confirm that this is so. The kinetics of RNA synthesis will also be shown to be consistent with the idea that there is a causal link between the stimulation of RNA synthesis and the stimulation of initiation of chromosome replication in *dnaA* strains.

The stimulating effect of chloramphenicol on the rate of DNA synthesis was confirmed with the system investigated in this thesis. Cultures of the *dnaA46* strain A3 and the *dna*$^+$ strain A3$^+$ were grown at 36.5°C in M9 casamino acids medium to A_{450} 0.2 and chloramphenicol was added to a concentration of 150 $\mu g ml^{-1}$. Samples were then taken for pulse labelling as described (section 2.II). The results are shown in Fig. 4.1. There is, as expected, a large and rapid increase in the level of incorporation of label into the A3 culture in agreement with the results of Messer *et al.* (1975). The rate of incorporation of label reaches a peak of about six times the pretreatment level. In a similar experiment, the level of RNA synthesis was measured in chloramphenicol treated and untreated cultures of A3, the results being shown in Fig. 4.2. There is again a large increase in the rate of incorporation of label. This effect is well known in *dna*$^+$ strains andthe data in Fig. 4.2 agree well with published findings on the rate of stable RNA accumulation after

Figure 4.1. DNA pulse labelling of A3 and A3$^+$ during chloramphenicol treatment

Pulse labelling of DNA was performed as described in section 2.II. Cultures were grown in M9 casamino acids medium at 36.5°C and chloramphenicol was added at time 0 (A_{450} 0.2).

- ● A3 treated with chloramphenicol.
- ▼ A3$^+$ treated with chloramphenicol.
- □ represents points calculated from the data in Fig. 4.3 as described in the text.

The culture used was the same as that from which samples were taken for hybridisation (see Figs. 4.4 and 4.5).

I am grateful to Dr E. Orr for performing the pulse labelling experiments and allowing me to use the results.

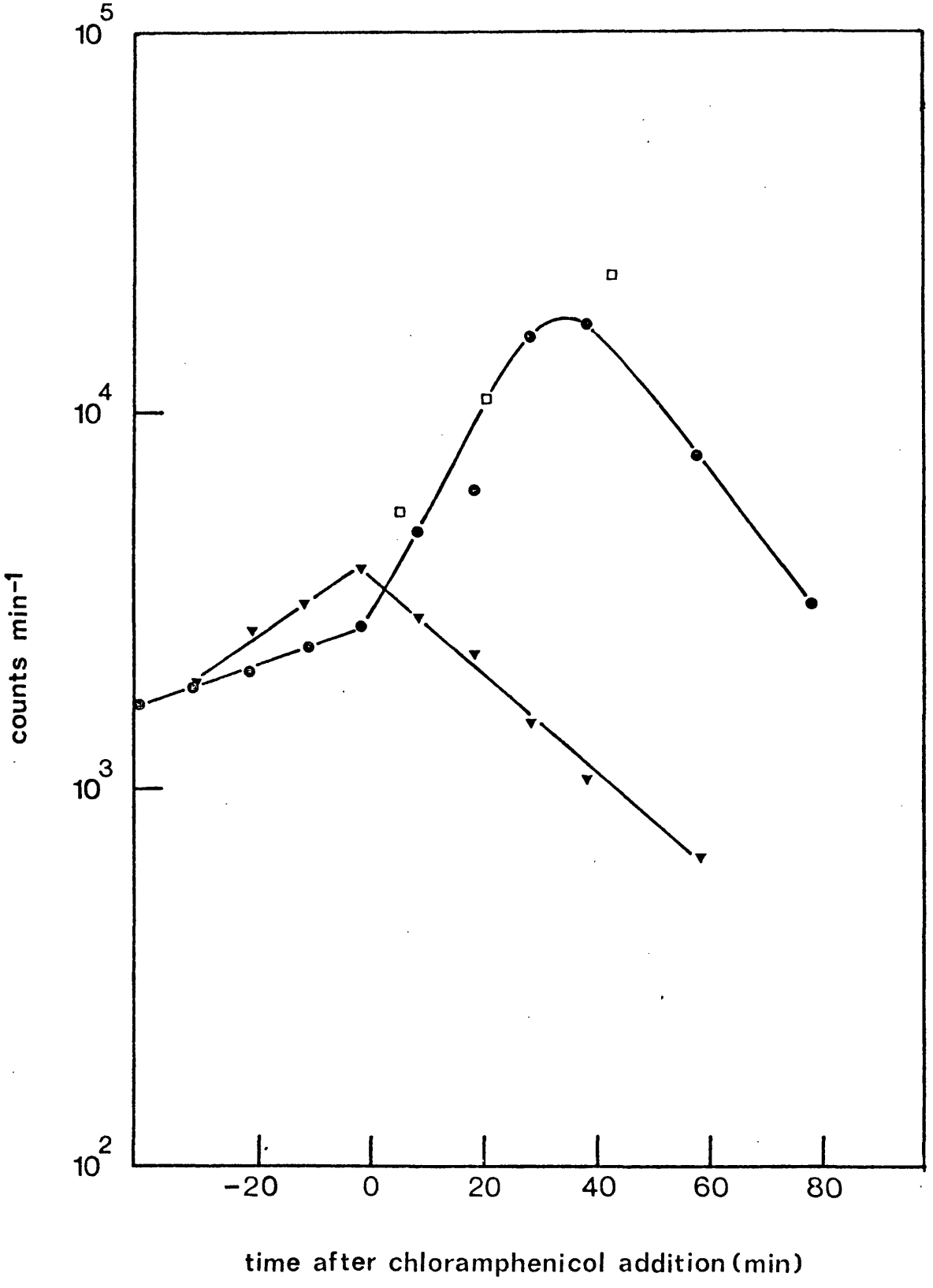

10^5
10^4
10^3
10^2
counts min-1
-20
0
20
40
60
80
time after chloramphenicol addition (min)

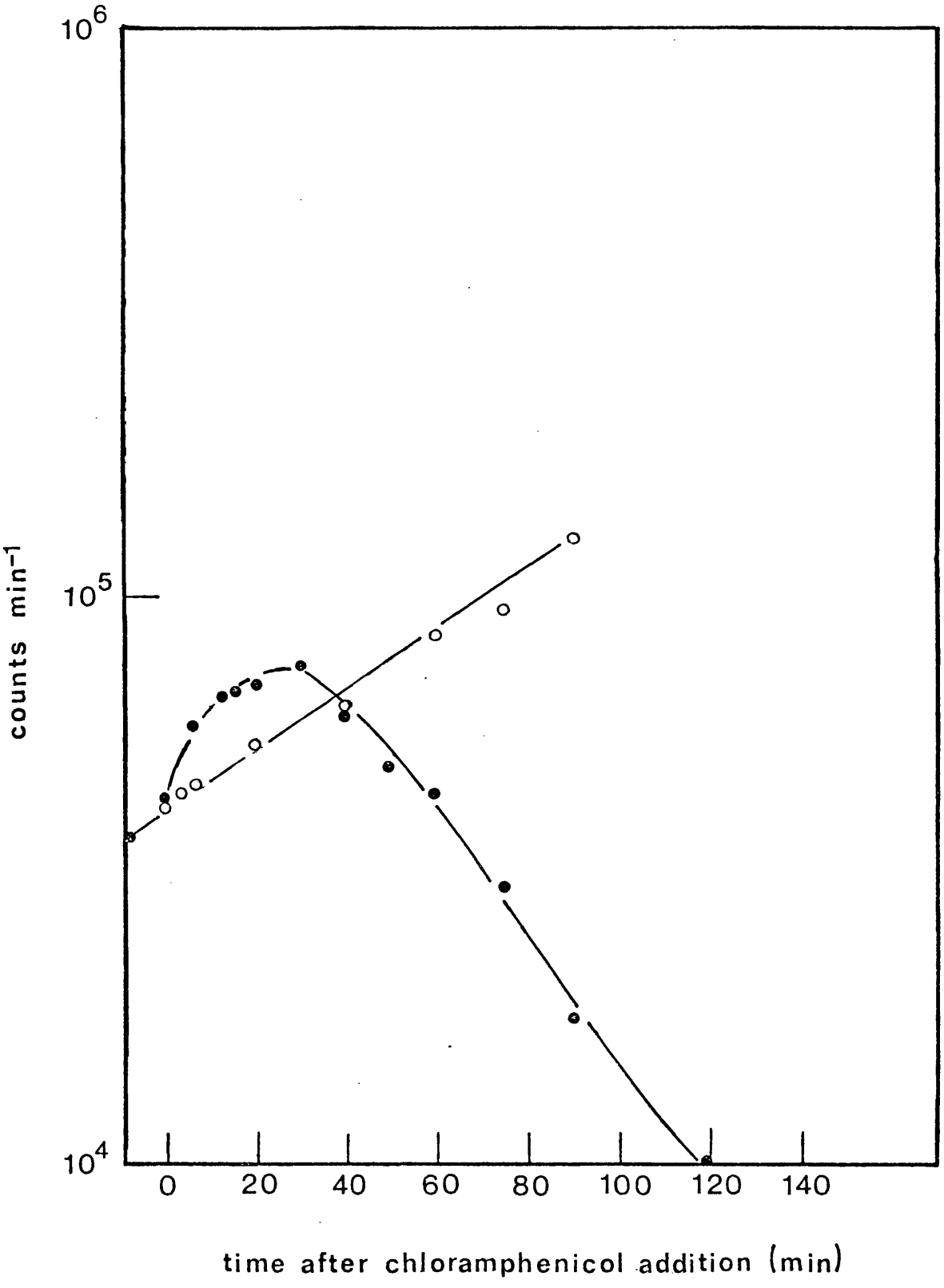
Figure 4.2. RNA pulse labelling of A3 during chloramphenicol treatment

Pulse labelling of RNA was performed as described in section 2.II. The cultures were grown in M9 casamino acids medium at 36.5°C and chloramphenicol was added at time 0 (A_{450} 0.2).

● A3 treated with chloramphenicol.

○ A3 untreated control.

I am grateful to Dr E. Orr for performing the pulse labelling experiments and allowing me to use the results.


10^6
10^5
10^4
counts min^{-1}
0
20
40
60
80
100
120
140
time after chloramphenicol addition (min)

chloramphenicol treatment (Midgley and Gray, 1971). Comparison of Figs. 4.1 and Fig. 4.2 shows that the stimulation of the rate of RNA synthesis is shorter lived than the stimulation of the rate of DNA synthesis. If the increased DNA synthesis were due to the initiation of extra cycles of replication, then the expected temporal pattern of initiation would be similar to that actually observed for RNA synthesis. The results therefore support the hypothesis of Orr et al. (1978) who proposed that the stimulation of the rate of synthesis of an RNA species which interacts with the dnaA product in initiation is responsible for the stimulation of the rate of initiation. Although the 4.1 and 4.2 shows that the stimulation in the rate of RNA synthesis is shorter lived. This is in agreement with the hypothesis of Orr et al. (1978) that it is the increased level of RNA synthesis that leads to the increase in initiation of replication in dnaA strains. Although the RNA stimulation in E.coli has been well documented by hybridisation as well as pulse and continuous labelling, the stimulation of DNA synthesis has only been studied by labelling of DNA so that two problems remain to be solved. Firstly the observed increase in DNA synthesis may not be due to increased initiation at the E.coli origin, though studies of the final level of DNA accumulation suggest that it is, (Messer et al., 1975; Hansen and Rasmussen, 1977; Orr et al., 1978). Secondly since radioactive labelling, and pulse labelling studies in particular are very susceptible to changes in precursor pool sizes in non steady state conditions (see O'Donovan, 1978) the pulse labelling data may not reflect the true kinetics of initiation. The rate of incorporation seen may have been exaggerated due to a sudden fall in precursor levels.

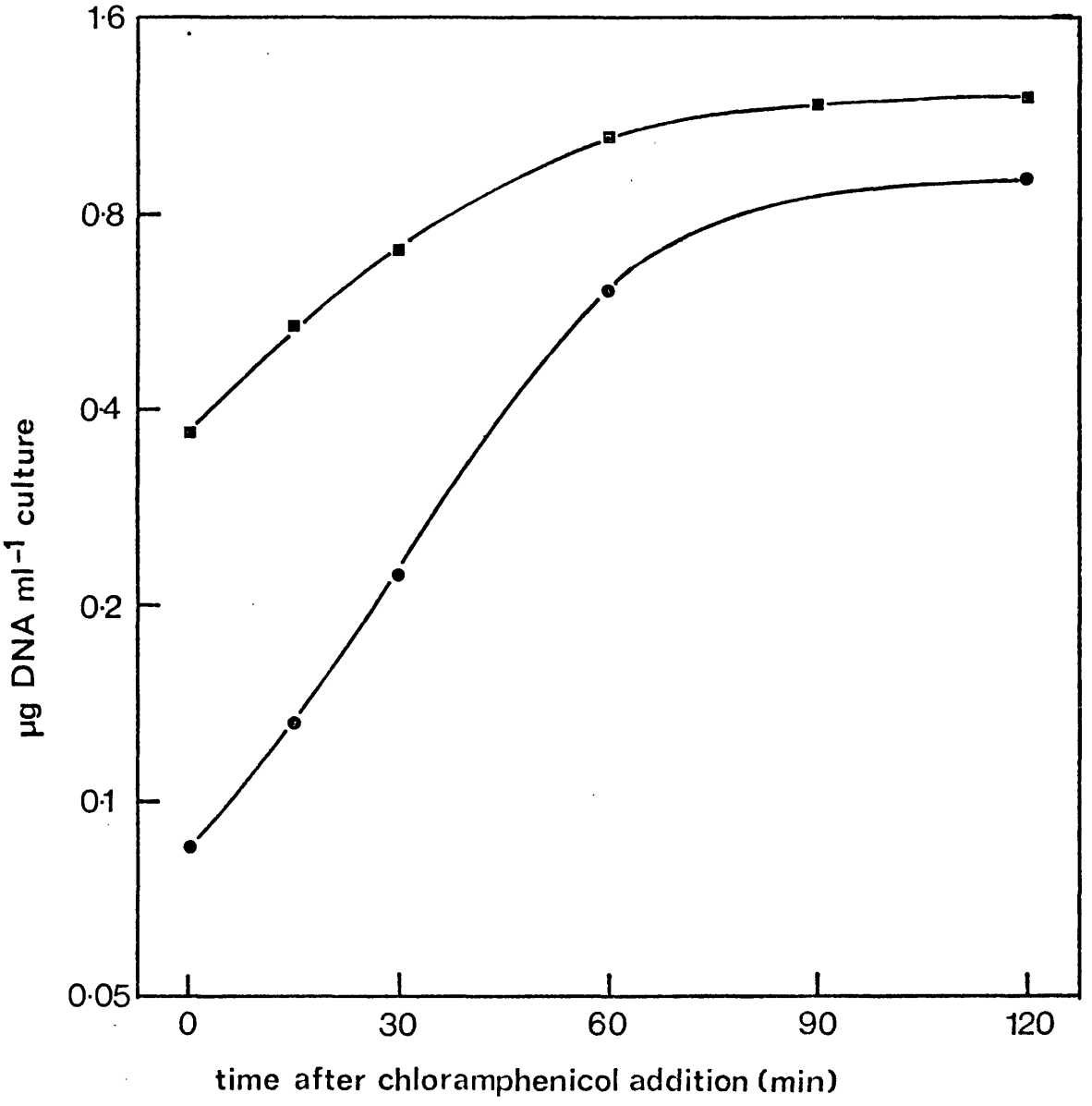
To eliminate the latter possibility the rate of DNA accumulation was estimated chemically. Cultures of A3 and $A3^+$ were grown as before. Chloramphenicol was again added at A_{450} 0.2 (t=0) and 50ml samples taken at the times indicated (Fig. 4.3) for the estimation of DNA content as described in section (2.II). Fig. 4.3 shows that in A3 DNA was synthesised at an apparently exponential rate, with a doubling time of 21 min for more than 30 min after the addition of chloramphenicol. This represented more than a doubling in the exponential rate of synthesis since A3 grew with a doubling time of 44 min under the growth conditions used in this experiment. Strain $A3^+$ (mass doubling time of 24 min) showed a gradual fall of the rate of DNA synthesis as expected if initiation was blocked. To check the validity of the data in Fig. 4.1, the mean changes in the DNA contents between time points in Fig. 4.3 were calculated, divided by the time over which the change occurred and treated as the best estimate of the rate of change at a time midway between the time points. These new points were then plotted for comparison on Fig. 4.1. For the purposes of normalisation the rate of DNA synthesis at t=0 was taken to be rising in proportion to the mass of the culture, and assumed to be equal to the rate of incorporation at t=0 in Fig. 4.1. Comparison of the two sets of data in this way shows that the pulse labelling data have not been grossly affected by changes in precursor pools and therefore give a fairly reliable picture of the kinetics of DNA synthesis.

In order to deduce the pattern of replication after chloramphenicol addition samples were taken for the analysis of marker ratios by DNA-DNA hybridisation. 100ml samples were taken from the A3 and $A3^+$ cultures used for the DNA pulse labelling at t= -20, 0, 5, 15, 30 and 90 min, t=0

Figure 4.3. DNA content of A3 and $A3^+$ cultures treated with chloramphenicol

Estimation of DNA content was performed by diphenylamine assay as described in section 2.II. The cultures were grown in M9 casamino acids medium at 36.5°C and chloramphenicol was added at time 0.

- ● A3 treated with chloramphenicol.
- ■ $A3^+$ treated with chloramphenicol.


1·6
0·8
0·4
0·2
0·1
0·05
µg DNA ml⁻¹ culture
0
30
60
90
120
time after chloramphenicol addition (min)

being the time of addition of chloramphenicol. DNA from each sample was purified, loaded onto filters and hybridised (as described in section 2.II) in triplicate to each of four probes : pLG4 (*argE*$^+$), pLG5 (*proA*$^+$), pLG18 (*manA*$^+$) and λ318 (*oriC*$^+$). The data (Figs. 4.4 and 4.5 and Table 4.4) were treated in the following manner. For each triplicate set of filters the mean number of counts binding was calculated, together with the standard error of the mean. This mean reading was then divided by the mean reading obtained with each of the other probes for the same DNA sample to obtain a set of ratios. Finally each ratio was normalised against the mean of the corresponding ratios for the two pretreatment samples.

If the observed burst of DNA synthesis upon addition of chloramphenicol to A3 (Fig. 4.1) was due to a burst of fresh initiation from the *E.coli* origin, the hybridisation experiment should reveal a rapid rise in the ratio of *oriC* to the other markers soon after the drug treatment. This is clearly revealed in Fig. 4.4 which shows the data for A3. If, in addition, the burst of initiation were to have given rise to a wave of replication forks travelling around the chromosome in the normal pattern (Bird *et al.*, 1972) and with the usual replication time we would expect the marker ratios to reflect this. The observed ratio of each marker to those farther from the origin was indeed seen to rise discretely at a time consistent with it being due to the "wave-front" of the higher density of replication forks reaching that part of the chromosome.

The measured *oriC*:*manA* and *argE*:*manA* ratios rose by 20%-40% after 15-30 min, whereas the data in Fig. 4.3 show that the rate of DNA synthesis doubled very soon after treatment. If we suppose that the rate of replication fork travel was not greatly affected, that doubling

Figure 4.4. Hybridisation analysis of marker ratios in A3 treated with chloramphenicol

A culture of A3 was grown for 5 generations at 36.5°C in M9 casamino acids medium and chloramphenicol (150 $\mu g ml^{-1}$) was added at A_{450} 0.5 (t=0). Samples were taken at the times indicated and hybridised as described in section 2.II and in the text. The data was treated as described in the text to obtain the following ratios:

● oriC:manA, ■ argE:manA, ▲ proA:manA, □ oriC:argE,

△ oriC:proA

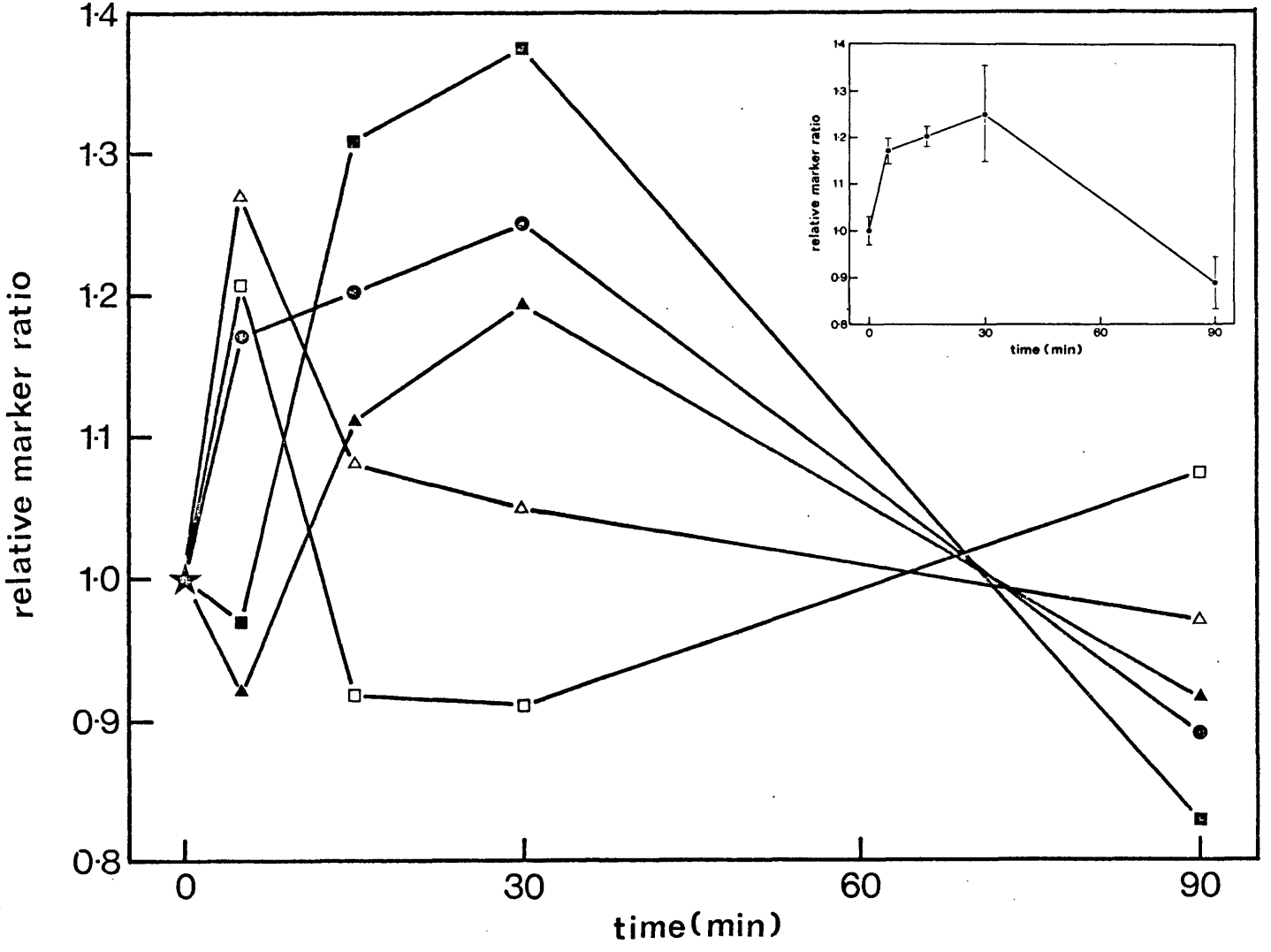

relative marker ratio
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0
30
60
90
time(min)
relative marker ratio
time (min)

Figure 4.5. Hybridisation analysis of marker ratios in $A3^+$ treated with chloramphenicol

A culture of $A3^+$ was grown and treated as described for A3 in Fig. 4.4.

● oriC:manA, ■ argE:manA, ▲ proA:manA

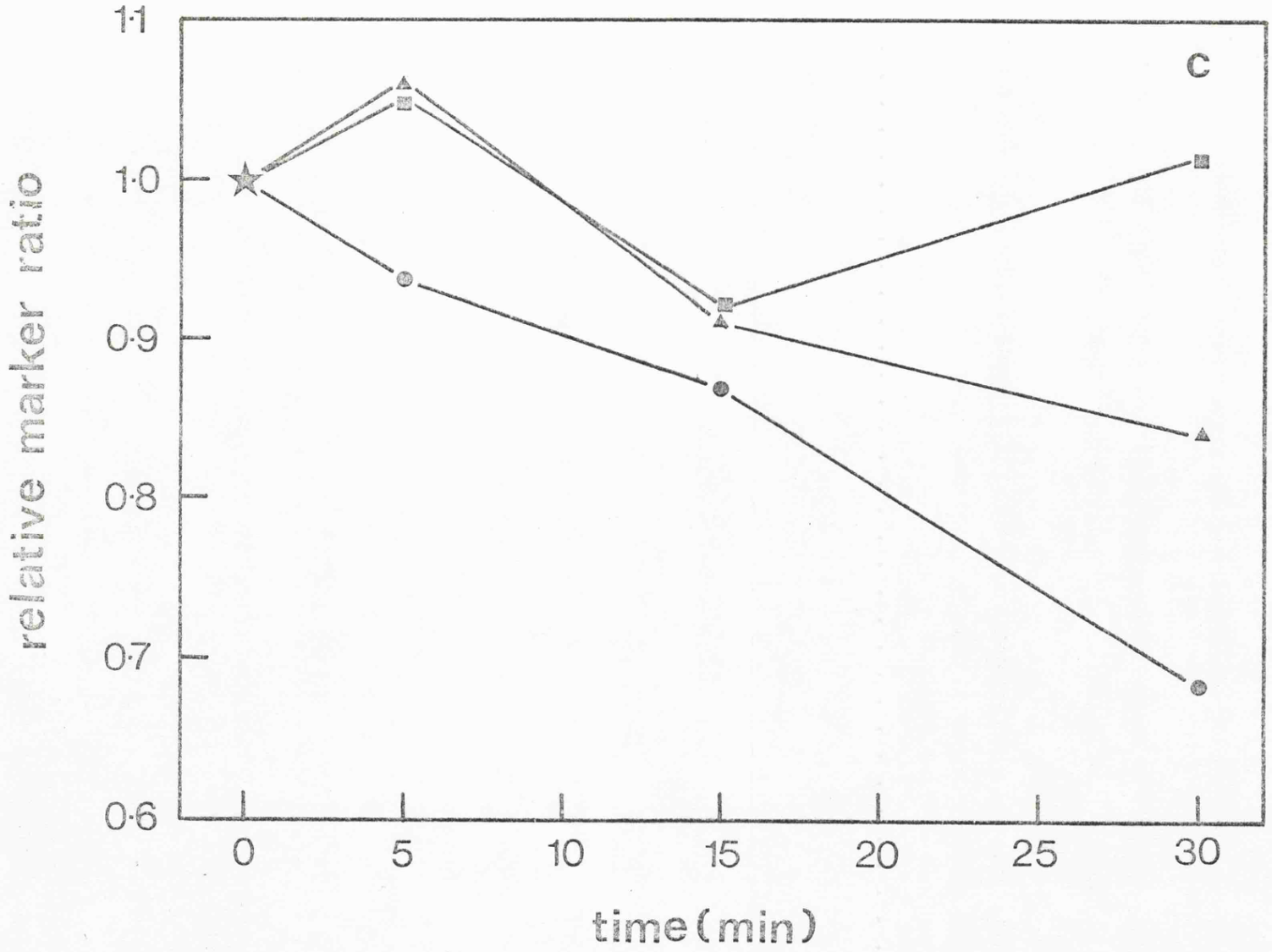

C
1·1
1·0
0·9
0·8
0·7
0·6
relative marker ratio
0
5
10
15
20
25
30
time (min)

Table 4.4. Ratios and standard errors of counts obtained for different marker probes hybridised against DNA from chloramphenicol treated A3 and $A3^+$

The table shows the ratios of the mean counts binding to different DNA samples together with the percentage standard error.

time	oriC/argE	oriC/proA	oriC/manA	argE/proA	argE/manA	proA/manA
			A3 samples			
-20	6.03± 3.6%	3.14± 1.4%	2.37±1.7%	0.520±3.9%	0.392±4.5%	0.754±2.3%
0	6.12± 2.8%	3.42± 4.4%	2.36±3.0%	0.557±5.4%	0.385±4.3%	0.690±5.9%
5	7.35± 4.7%	4.18± 2.2%	2.77±2.4%	0.568±4.6%	0.376±5.2%	0.663±2.6%
15	5.59± 4.4%	3.56± 5.4%	2.84±1.8%	0.637±9.3%	0.508±5.6%	0.797±6.8%
30	5.54± 8.6%	3.45±13.4%	2.95±8.5%	0.623±7.9%	0.533±3.4%	0.855±8.3%
90	6.53±11.4%	3.19± 5.9%	2.10±6.3%	0.488±7.4%	0.321±8.6%	0.656±3.0%
			$A3^+$ samples			
-20	4.88± 3.0%	4.95± 2.4%	5.17±4.3%	1.015±4.1%	1.059±6.0%	1.044±5.5%
0	5.01± 2.4%	4.64± 3.0%	4.65±4.1%	0.926±3.0%	0.977±4.0%	1.001±4.7%
5	5.20± 2.4%	5.08± 2.0%	4.61±3.8%	0.978±1.6%	0.886±3.4%	0.906±3.0%
15	4.55± 1.5%	4.37± 1.2%	4.26±2.9%	0.961±1.7%	0.937±3.4%	0.975±3.1%
30	5.03± 2.5%	4.03± 2.9%	3.35±2.4%	0.801±1.4%	0.665±0.9%	0.836±1.3%

in rate must have been due to a doubling in the number of replication forks. Before the treatment, the replication time probably being similar to the generation time of 44 min, there would have been one replication fork per chromosome terminus and an oriC:terC ratio of 2:1. A doubling in the number of replication forks would have caused a rise of 50% in this ratio from 2:1 to 3:1 initially, and since the rate of DNA synthesis continued to rise a rather greater change in the ori:ter ratio would be expected later. Nonetheless this is not inconsistent with the hybridisation data since they include a nonspecific background element that will tend to cause the magnitude of changes in marker ratios to be underestimated, (see section 3.III). This background may well have been larger for λ318 than for pLG4, because it contains more heterologous sequences, so that the oriC:man ratio would have been affected more than the argE:man ratio.

If the addition of chloramphenicol caused little or no change in the replication time of the chromosome (and the data in Fig. 4.4 are not consistent with a large change) then the termination of pre-existing cycles of replication would have continued for some time (at least 40 min). Thus the number of chromosomes would have continued to rise at the old growth rate. In addition, the continued rise in the ratio of proximal: terminal markers indicates that following the initial burst of initiation, initiation continued at a level higher than the pretreatment level and possibly at an increasing rate. The hybridisation data are therefore not inconsistent with the continued pseudo-exponential rise in the rate of DNA synthesis seen in Fig. 4.3 as the number of chromosomes was increasing exponentially at the old growth rate, in addition to which, the number of replication forks per chromosome rose suddenly and continued to rise for perhaps 30 min.

All cycles of replication initiated before the addition of chloramphenicol would have terminated before the 90 min sample was taken so that there is no clear reference to aid interpretation of the data. It may nonetheless be clearly seen that the marker ratios had fallen to the pretreatment level, or in the case of the important proximal: terminal marker ratios to well below the pretreatment level. Therefore, although all of the replication forks may not have terminated, initiation was either at a very low level or most probably had ceased.

With the aid of Fig.4.3 we may therefore conclude that the initiation rate rose for at least 30 min, that initiation had effectively ceased after 90 min and that it was probably at a much reduced level after 60 min (Fig. 4.3). The kinetics of initiation seem, then, to be in good agreement with the kinetics of RNA synthesis seen in Fig. 4.2. This suggests strongly that there is a link between the two and supports the hypothesis of Orr et al. (1978).

The results of the control hybridisation experiment using the chloramphenicol treated $A3^+$ DNA samples show clearly that the ratios of proximal:terminal markers fell progressively over 30 min. This is consistent with the cessation of initiation and the termination of existing cycles of replication.

The overall amount of DNA synthesised by A3 (Fig. 4.3) was shown finally to have approached that of the $A3^+$ culture. The ratio of the DNA contents of the two cultures (which was equal to the DNA/mass ratio since the two cultures were of the same absorbance) was 4.4:1 at 36.5°C before the addition of chloramphenicol in agreement with published figures for this strain obtained in a different way (Pritchard et al., 1978; Orr

et al., 1978). After 120 min the difference was reduced to 1.35:1, and is only 1.15:1 if corrected for the difference in the absorbance of the cultures after 120 min (0.25 and 0.29 for A3 and $A3^+$ respectively). The DNA:mass ratio therefore appears to have approached the wild type level. The significance of the size of this change is unclear (see section 5.III) but it is clear that it is connected with the expression of a latent "initiation capacity".

CHAPTER 5

5.I. A TECHNICAL SUMMARY

The production of a set of recombinant plasmids carrying DNA fragments from various parts of the E.coli chromosome was a major part of this study, in the technical sense. The inability to obtain plasmids carrying trp, gal and his has been discussed in section 3.II. This was in part due to the length of time needed for the screening, which meant that it was not possible to test a sufficient number of clones to have a 99% chance of obtaining any given EcoR1 fragment. The indirect screening method used was laborious and a direct selection would possibly have been much more economical of time and effort. The choice of recipient for the original transformation, although made for seemingly good reasons, proved to be a mistake; a recA$^-$ strain, and perhaps an F$^+$ rather than F$'$ strain would have been preferable. Nonetheless, a number of chromosomal fragments were isolated which according to searches have not been reported in the literature previously. Unfortunately it was necessary that E.coli DNA fragments inserted into vectors not homologous with RSF2124 be used as hybridisation probes. This might have been a problem were it not for the fact that all of the experiments were designed to measure comparative marker ratios rather than absolute values.

The presence of a large nonspecific "background" element in the hybridisation experiments was one of the most serious technical problems encountered during the course of this study. Bird et al. (1972) used 'phage DNA to probe DNA isolated from multiply lysogenic strains. The use

of non-lysogens as controls enabled them to make a reasonably direct estimate of the background binding in each experiment and to correct for it in their calculations. Since the equivalent in terms of the system used in this study (E.coli strains containing exact deletions of the parts of the chromosome represented on the recombinant plasmid) were not available for most of the probes, the experiments undertaken as part of this study had necessarily to be performed in a way that controlled internally for the "background". This limited the type of experiments that could be undertaken. Attempts to eliminate the background were unsuccessful though more stringent conditions did reduce it somewhat according to indirect estimates. There seems to be no way of overcoming the problem with the system as it stands. RNA probes are known to give a lower background than DNA probes. It would be interesting to see whether the use of in vitro transcription to produce labelled RNA copies of the plasmid probes would yield cleaner results than the use of the "nick translated" DNA probes.

The method of Southern (1975) as performed by Jeffreys and Flavell (1977) did not suffer from the problem of background, but proved to be not strictly quantitative. This could have been due to nonquantitative blotting or because of the different temperature and buffer system used for the hybridisation as compared with the other hybridisations reported in this thesis, or may be inherent in the varying DNA concentration on the filter. The use of dextran sulphate resulted in more efficient, but still non-quantitative, hybridisation. Other modifications, such as depurination of the DNA after electrophoresis and the use of diazo-benzyloxymethyl paper have been reported as improving the transfer of large and small

fragments (Wahl et al., 1979), and as the methods continue to be improved, their use in quantitative experiments maybe worthy of future investigation. The use of a formamide buffer system for hybridisation could also be an improvement.

Despite the background problems which limit experimental design, the original aim of producing a versatile hybridisation system has been largely achieved. It is no longer necessary to synthesise lysogenic derivative strains in order to investigate replication, thus any set of strains maybe readily compared for differences in marker ratios and an experiment performed on one strain (e.g. A3) may be readily repeated on another (e.g. $A3^{+}$).

5.II. ON THE CONTROL OF INITIATION AS REFLECTED BY REPLICATION IN Hfr STRAINS

The first set of experiments presented in this thesis, were designed to find out whether the origin of replication of the F plasmid could function when in the integrated state under normal steady state physiological conditions. Although it has long been assumed that the F origin does not function in the integrated state, the evidence was poor and contradictory (see section 1.IV). There are three possible alternatives for the control of F replication in Hfr strains; (a) the initiation of the F origin may be completely blocked by some special mechanism, (b) the control of F initiation may be inactivated giving overinitiation or (c) the control of initiation may be unaffected and may continue as it would in the autonomous state. The second alternative is easily dismissed since over-initiation would have easily been detected by any of the many studies undertaken so far (see section 1.IV) and would probably have profound, and potentially lethal, effects upon the metabolism of the host cell. Any blocking of the F origin of replication due to recombinational inactivation of an essential gene during integration of the F plasmid is ruled out since most Hfrs (including KL99 and AB313) are known to give rise to F' plasmids (Low, 1972). The existence of type II F' plasmids in which the F plasmid has excised from the chromosome together with the flanking chromosomal sequences on both sides (Low, 1972) confirms that no site-specific back-recombination is necessary to regenerate an essential structure during F' formation. Any special switch off of the F origin as in alternative (a) above would therefore involve some sort of repression

or steric inhibition. The finding of Chandler et al. (1976) that the F plasmid origin of replication is active only following thymine limitation, could be consistent with such a special repression mechanism. The results given in section 4.I, however, are best interpreted as showing that the KL99 origin is still active under exponential steady-state conditions. The initiation of the F origin in Hfr strains must therefore be controlled in the same way as the initiation of autonomous F replication.

What is the nature of this control? Models for the control of initiation may be divided into two classes (see section 1.II) and the two classes lead to two different predictions about the control of the replication of cointegrates (see section 1.IV). Briefly, group A models rely upon the proposition that the rate of initiation is controlled, rather than the origin concentration (or copy number) so that in an Hfr strain the F origin should initiate as frequently as it does in the autonomous state. Group B models rest upon the premise that the origin concentration is regulated, so that in an Hfr the gene dosage of F will always be at least as high as in the autonomous state. Where the F plasmid is integrated at some distance from the origin, such that the time taken for replication forks to move from the origin to the F plasmid is of a similar order to the generation time of the culture, then both types of model lead to similar predictions about the "copy number" of F in the integrated state. This is because the replication forks from the E.coli origin and the F origin will meet at some point between the two and the cycles of replication will not be additive. The "copy number" of F is therefore expected to be similar to that of an autonomous F plasmid in each case (or similar to that part of the chromosome into which it is integrated if that is higher).

As discussed in section 1.IV, where the F plasmid is integrated very close to the E.coli origin, the replication forks from one origin will probably have already passed the other origin by the time any initiation occurs there. Consequently the forks will not meet and the cycles of replication will be additive, as in small cointegrates. Thus in this situation a type A model leads to the prediction of a gene dosage at the E.coli origin equal to the sum of those of the E.coli origin and the F origin and thus a raised gene dosage for the whole chromosome and a greatly increased DNA/mass ratio. A type B model would predict a "copy number" for the origin region equal to that for the origin with the lower initiation mass in the autonomous state. The values of DNA/mass for Hfr AB313 and its isogenic F^- strain presented in section 4.I support a type B model rather than a type A model since the DNA/mass ratio was not seen to be greatly changed. The findings of Chandler et al. (1976) that there was little or no replication from the F origin in a derivative of Hfr OR11 is difficult to interpret in this respect since the point of insertion of F in that Hfr is half way round one replication arm of the chromosome. With a generation time of only 26 min in their experiment the predictions of type A and type B models would not be clearly dissimilar.

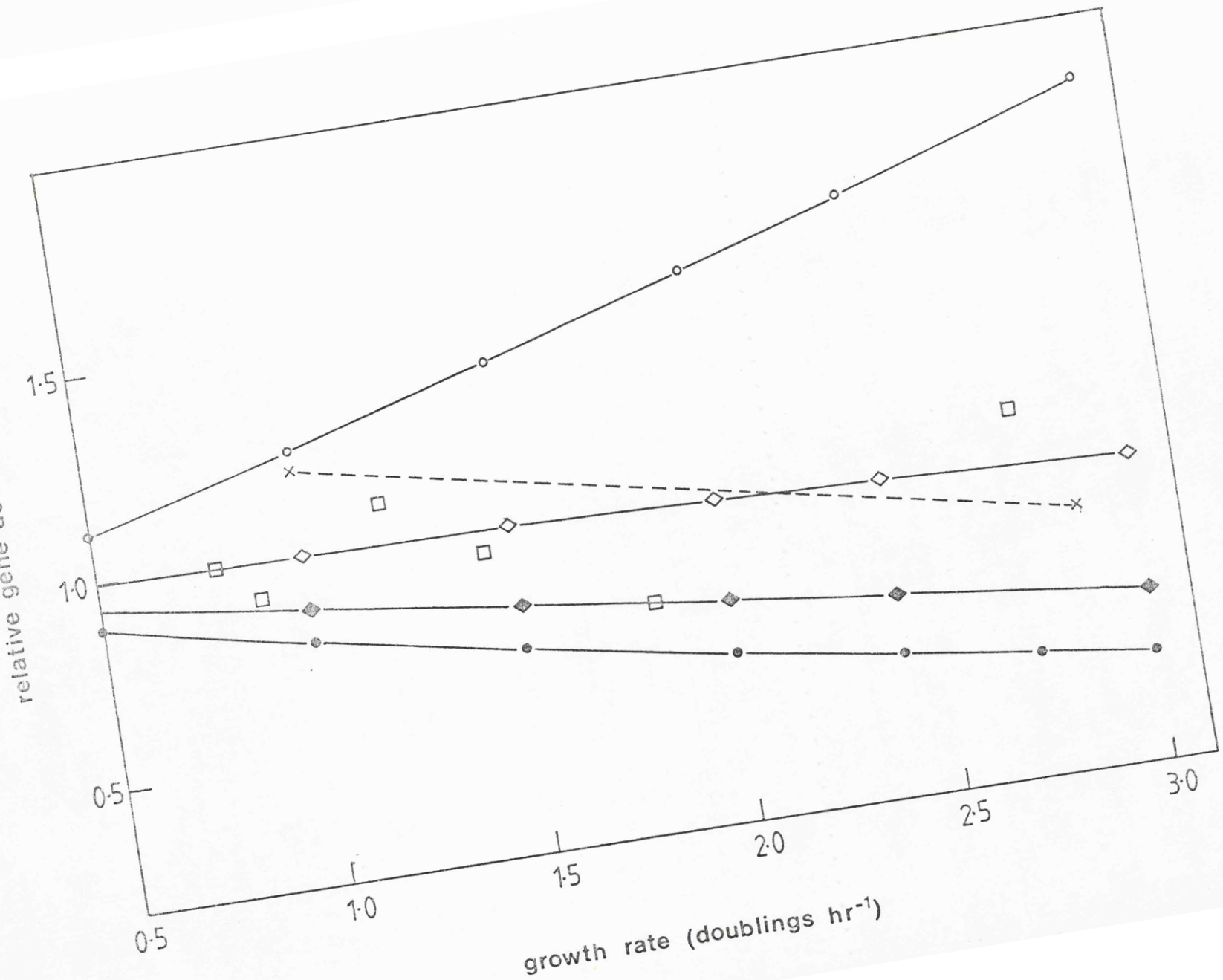
The difference between the results of Chandler et al. (1976) and those presented here concerning origin usage in exponentially growing Hfr cultures may be explained as a reflection of the different gene dosages of the regions of the chromosome into which F is integrated in the two Hfrs used, relative to that of autonomous F at the same growth rate. Data on the copy number of F at different growth rates obtained in two independent studies by different methods (Collins and Pritchard, 1973; Pritchard et al., 1975) are not in close agreement (see Fig. 1.1). The experimental points

from Pritchard et al. (1975) had been calculated using an estimate of the distance between oriC and lac which is no longer accepted. I have re-calculated the data using the latest estimates of the map positions (Bachmann and Low, 1980). The recalculated values agree more closely with the data of Collins and Pritchard (1973) and are shown in Fig. 5.1 together with the calculated values for the relative gene dosages of oriC, terC, lac (near to which F is integrated in OR11, the Hfr used by Chandler et al. (1976), and pyrC (which is transferred early during mating by Hfr KL99). The difference between the findings for KL99 (section 4.I) and those reported by Chandler et al. (1976) for OR11 is entirely in agreement with the hypothesis that the F plasmid when in the Hfr state is regulating its copy number as it would in the autonomous state.

A type B (origin titration) model explains better than a type A (initiation frequency) model, the data of Cabello et al. (1976), who found that in a ColE1-pSC101 cointegrate, pSC134, replication occurred solely from the ColE1 origin i.e. the origin with the lower initiation mass (see section 1.IV). Cointegrates composed of ColE1 or pSC101 and the origin region of plasmid R6K are possibly the best studied cointegrates (Kolter and Helinski, 1978; Crosa et al., 1978) and their properties are strongly in accord with a mass titration mechanism. R6K has naturally two origins, designated oriα and oriβ. The αori seems to have the lower initiation mass since it is the dominant origin in the natural plasmid (copy no $\sim$ 20). Derivatives containing only oriα still have a copy number of 20 whereas those containing only oriβ have a copy no. of 10. Thus the copy numbers are not additive. Again when either or both of these origins is joined to ColE1 or pSC101 the final copy number is equal to that of the component

Figure 5.1. The relative gene dosage of autonomous F recalculated

The relative gene dosages of oriC (○), terC (●), lac (◇), and pyrC (◆) were calculated as described in the legend to Fig. 1.1. The data for the relative gene dosage of the autonomous F plasmid are redrawn from Collins and Pritchard (1973) (×) or are calculated from revised estimates of $^{F}/_{oriC}$ (□). The values of $^{F}/_{oriC}$ were recalculated from the β galactosidase levels given by Pritchard et al. (1975) as described by them. Values of x = 0.487 and C = 40 min were used in these calculations x being a measure of the distance between lac and oriC compared with that between terC and oriC while C is the chromosome replication time. The increased values of $^{F}/_{oriC}$ result mainly from the change in the value of x from that of ~ 0.64 used by Pritchard et al. (1975). This change is due to the more accurate mapping of oriC in the last few years (see Bachmann and Low, 1980).


1·5
1·0
0·5
relative gene dosage
0·5
1·0
1·5
2·0
2·5
3·0
growth rate (doublings hr⁻¹)

having the highest copy number in the autonomous state. Taken together these plasmid studies indicate that a mass titration control may be common to most bacterial replicons. This is especially significant as ColE1, pSC101 and R6K are relatively small plasmids, the first two showing unidirectional replication (Lovett et al., 1974; Cabello et al., 1976; Kolter and Helinski, 1978; Crosa et al., 1978), whereas F and the E.coli chromosome seem to have bidirectional replication (Eichenlaub et al., 1977; Prestcott and Kuempel, 1972; Bird et al., 1972). Whether unidirectionality or bidirectionality of replication is a truly significant characteristic is called further into question by the finding that ColE1 may exhibit bi-directional replication, with initiation in the second direction occurring after a delay, as is also found in R6K (Kolter and Helinski, 1978; Crosa et al., 1978).

Given that a type B model fits the weight of evidence most closely, it is not possible to make a definite choice between different type B models. The inhibitor dilution model (Pritchard, 1968; Pritchard et al., 1969; Pritchard, 1978) is the best described of these models and can account for all of the observations concerning the copy number of F. The slight rise in DNA/mass ratio found in AB313 relative to the isogenic F^- strain may well not be significant but if it is a genuine effect it could indicate that the control mechanism is not stringent. In terms of the inhibitor dilution model, the dilution of inhibitor with growth would cause a gradual rise in the probability of initiation as suggested by Pritchard (1978) rather than there being a critical concentration for initiation. Since the relationship between the inhibitor concentration and the probability of initiation with time will depend upon the stoicheiometry

of the reaction it will probably be different for different replicons, thus explaining the change in F/oriC ratio with growth rate (Pritchard, 1978). The change in the plasmid/origin ratio with growth rate can also be explained by an effect of growth rate on the size of an inhibitor pulse, or in the case of the unstable inhibitor model (Pritchard et al., 1969) by a change in the rate of inhibitor synthesis.

Not all of the reports in the literature seem to support a type B model. Nordstrom and co-workers (Engberg and Nordstrom, 1975; Gustafsson et al., 1978a; Gustafsson, 1977; Gustafsson and Nordstrom, 1980) state that plasmid R1 replication is regulated at a constant rate at all growth rates, and show that there is an immediate change to a new rate of replication relative to the rate of synthesis of protein during a nutritional shift. This would seem to be most consistent with the idea that there is an initiator of replication subject to autoregulation (Sompayrac and Maaløe, 1973; Hansen and Rasmussen, 1977). As already discussed, according to inhibitor models, a change in the size of an inhibitor pulse, or in the probability of initiation occurring at a derepressed origin (perhaps due to a change in the concentration of a secondary reactant c.f. dnaA section 5.III) would lead to a change in the concentration of a replicon at different growth rates. This change could be of such a magnitude as is necessary to produce a constant rate of replication at all growth rates. The inhibitor dilution model in the latter case, and the unstable inhibitor model in both cases will however predict an initial overcompensation during a shift in growth rate. The one exception is the case of the inhibitor dilution model if it is assumed that the size of the plasmid inhibitor pulse is increased with increasing growth rate such as to keep the rate of replication constant.

This model would lead to a prediction of an immediate change in the rate of replication relative to the rate of protein synthesis for a multicopy plasmid selected at random for replication, just as seen by Nordstrom and co-workers. In this case it is necessary to suppose that the rate of synthesis of chromosome inhibitor is not affected by the growth rate. Thus the above results do fit with a type B model but only under certain very definite circumstances.

Watson and Scaife (1980) have very recently reported that integrated and autonomous forms of RP4 can coexist stably in the same cell. This is in contrast to the situation with the two forms of F, which are found to be mutually incompatible (Scaife and Gross, 1962). It seems that in the case of RP4 there may be some special mechanism whereby the incompatibility property is repressed in the integrated state. Whether the replication functions are also "switched off" raises the question of the relationship between replication functions and incompatibility. Most of the available evidence suggests that incompatibility cannot be lost without affecting replication properties though the converse is not true . Cabello *et al.* (1976) found that cointegrates of ColE1 and pSC101 in which there seems to be no replication from the pSC101 origin still show incompatibility towards pSC101. Iordanescu and Surdaneau (1979) have found that the staphylococcal plasmid pT181 still shows incompatibility when integrated into another plasmid, and moreover even a replication mutant in non-permissive conditions will show incompatibility when maintained by another replicon. These results agree with the findings of Meacock and Cohen (1979) who have reported that *rep* mutants are not always affected in their *inc* properties but *inc* mutants are always affected in their *rep* properties.

The RP4 derivative used by Watson and Scaife (1980) was not affected in its replication in that it could recombine out of the chromosome and maintain itself autonomously, at which time it regained its incompatibility. It would be interesting to find out whether RP4 can replicate in the integrated state. Certainly if it were titrating its copy number, the integrated form would probably not reach its autonomous concentration due to passive replication within the chromosome. Although the copy number is not certain it is certainly higher than that of F (R. Kolter personal communication) so that autonomously replicating plasmids could exist in the same cell from the copy number point of view. However, why the incompatibility property should only be expressed in the autonomous form is difficult to explain. Any segregation of the autonomous form of the plasmid would leave the host chromosome with an integrated "high copy number" plasmid which would probably have deleterious effects. Since the growth rate of the host is unaffected by the presence of the two forms of the plasmid (M.D. Watson personal communication) any segregation is ruled out, especially since two autonomous RP4 plasmids segregate extremely rapidly (M.D. Watson personal communication). De Vries *et al.* (1975) found an *inc* mutant of an Hfr strain with similar properties in that it could regenerate an autonomous plasmid that was *inc*$^{+}$. Interestingly Ogura *et al.* (1980) have reported that a mini-F-*oriC* composite replicon is stably maintained in Hfr cells of *E.coli*. They have proposed that replication is under the control of *oriC* but that the F partition mechanism ensures stable inheritance.

5.III. ON THE ROLE OF THE *dnaA* GENE PRODUCT IN INITIATION

The results reported in section 4.II confirmed by several different methods that there was extra DNA synthesis upon the addition of CAM to a *dnaA46* strain grown at an intermediate temperature. They also showed that this was due to reinitiation at the normal origin of replication *oriC* and that the resulting cycles of replication were normal in pattern and replication velocity. It therefore seems reasonable to conclude that such initiation is not completely anomalous, but rather reflects some properties of the initiation control system of *E.coli*. The finding that the DNA/mass ratio of the mutant approached that of the wild-type after CAM treatment of them both supports the supposition that the extra initiation was due to the release of some block of initiation, allowing the expression of a latent "capacity to initiate" in the mutant.

A number of authors have proposed such ideas (Messer *et al.*, 1975; Hansen and Rasmussen, 1977; Orr *et al.*, 1978) though there have been only estimates of the increase in the DNA content of the culture to confirm that the extra synthesis is due to reinitiation (Hansen and Rasmussen, 1977; Orr *et al.*, 1978; Tippe-Schindler *et al.*, 1979). It is the nature of the initiation capacity that is most in dispute. Orr *et al.* (1978) have proposed that initiation is blocked in *dnaA* strains at some stage of initiation subsequent to the normal initiation controlling step. This later step, which is proposed to be involved in transcription or in the action of an RNA species would become rate limiting in *dnaA* strains at the partially permissive temperature, giving rise to an accumulation of origins at which the first step in initiation has occurred. These origins would represent "initiation capacity".

/

Hansen and Rasmussen (1977) have proposed that the *dnaA*$^+$ gene product is a positive initiator of replication which controls its own synthesis by autoregulation. Denaturation of the *dnaA* protein would lead to a loss of this regulation and over production of the gene product. They further proposed that the active form of the protein is a multimer and that it is rendered inactive if only one of the identical subunits is denatured. Thus when protein synthesis was inactived by chloramphenicol there would be a capacity to initiate in the form of a large pool of *dnaA* product which could slowly renature and allow initiation to continue. They showed that the amount of residual DNA synthesis was greater if the temperature was shifted down to 28° upon the addition of CAM. This fits well with their hypothesis since the *dnaA* protein would more readily renature at 28° than at say 35°. There are, however, several important points not explained by this hypothesis : most notably it cannot account for the increase in the rate of initiation upon CAM addition at constant temperature, nor can it explain why amino acid starvation does not have the same effect as CAM in *dnaA46* strains. Further, if *dnaA* protein were the primary initiation control function it would be expected that the presence in the cell of plasmids such as pSC101 and F, which require *dnaA* product for replication (Hasunuma and Sekiguchi, 1977, 1979; Frey *et al.*, 1979; Tresguerres *et al.*, 1975) would have a serious effect on the normally precise timing (Koppes *et al.*, 1978) and level of initiation.

Tippe-Schindler *et al.* (1979) have proposed a model similar to that of Orr *et al.* (1978) in that the limiting factor in initiation is an RNA. They proposed that the synthesis of this RNA is controlled by an unstable repressor and that *dnaA* protein acts as an antirepressor. They then

explain the stimulation of initiation by CAM as being due to the inhibition of repressor synthesis. To explain the lack of such a stimulation in *dna*$^+$ strains they proposed a secondary control on the synthesis of other substances participating in the initiation reaction. It is a pool of such substances that represents "capacity" in their model. This model, unlike that of Orr *et al.* (1978) leads to the prediction that any means of inhibiting protein synthesis such as amino acid starvation, should stimulate initiation in *dnaA* strains which have an accumulated "capacity" to initiate". It is clear that in *dnaA46* strains amino acid starvation does not stimulate initiation except when the strains also carry the *relA* mutation (Orr *et al.*, 1978) but conflicting claims have been made for the *dnaA5* mutation. Messer *et al.* (1975) claim to have found a stimulation with amino acid starvation in an *E.coli* B/r background whereas no such stimulation has been detected in an *E.coli* K12 background (E. Orr, personal communication).

It seems on the whole that the results presented here fit best with the model of Orr *et al.* (1978) since the kinetics of initiation clearly follow the kinetics of RNA synthesis in strain A3. Whether the final comparative levels of DNA observed after CAM treatment of A3 and A3$^+$ is of biological significance, or merely fortuitous is not clear. The DNA/mass ratio of several *dnaA* strains has been shown to be inversely related to the growth temperature (Hansen and Rasmussen, 1977; Fralick, 1978a,b; Pritchard *et al.*, 1978). Hansen and Rasmussen (1977) have presented data which allow the deduction that the final DNA/mass ratio always rises to the same level at all growth temperatures above 28°C when there is no change in the culture temperature following CAM addition. Such a finding

is a requirement of the model of Tippe-Schindler et al. (1979), but not of those of Hansen and Rasmussen (1977) and Orr et al. (1978). When the temperature was shifted to 28°C following CAM addition however, Hansen and Rasmussen (1977) found that the stimulation in initiation was always greater than that needed to make up the deficit in DNA level compared with the level in cultures grown at 28° throughout. This is in agreement with the hypothesis of Orr et al. (1978). If it is supposed that the dnaA protein acts before that step in initiation at which a negative control factor is made (Pritchard et al., 1969) then initiation occurring after CAM addition will not lead to a negative feedback and the only control on initiation will be such factors as the length and level of RNA synthesis, the amount of dnaA product in the active form and the level of precursors. In such a case, the final level of DNA synthesis could not be predicted, but would be greater when the capacity was expressed at a lower temperature, since more active dnaA product would be present. Such an effect of the temperature at which capacity is expressed might also be explained in another way. Kellenberger et al. (1978) have found that certain revertants of dnaA strains are cold sensitive due to overinitiation of replication at the low temperature. The original dnaA46 strain is also cold sensitive (Orr et al., 1978). Although in this case it has not been demonstrated that the sensitivity is also due to overinitiation it could explain the extra stimulation seen at low temperatures.

It is perhaps instructive to speculate on why the stimulation of RNA synthesis should stimulate initiation in dnaA strains. It is known that RNA is involved in initiation since initiation as a whole (Lark, 1972) and the dnaA mediated step in particular (Zyskind et al., 1977) is sensitive

to rifampicin, and because a copy number mutation in E.coli has been mapped in the rpoC gene (Atlung, 1979). The finding that dnaA mutants are more sensitive to rifampicin than dna$^+$ strains is further evidence for the interaction of dnaA with this RNA mediated step in initiation (Orr et al., 1978). dnaA product could interact directly with an RNA. In such a case dnaA product may be a post transcriptional modifying enzyme, or the RNA may act like a ribosomal RNA, holding several proteins, of which dnaA protein maybe one, in the correct conformation to form an initiation complex. Alternatively dnaA product may act to regulate the synthesis of an RNA species. The location in the rpoB gene of mutations suppressing the DnaA phenotype (Bagdasarian et al., 1977) supports this alternative. A number of proposals for the mode of action of dnaA have already been made : among them that it is like the λ N gene product and acts as a transcriptional antitermination protein (Bagdasarian et al., 1978) or that it is ρ-like causing termination of transcription at the correct site (Atlung, 1979). Support for the former is seen in the finding that RNA polymerase core enzyme detaches from the folded chromosome at high temperature in a dnaAts strain. One may propose other ideas : for instance dnaA may act like Tu-Ts factor (Travers et al., 1970) and cause preferential transcription of one species of RNA. This last suggestion is in keeping with the observed effect of CAM on RNA synthesis and initiation since CAM preferentially stimulates the synthesis of stable RNA species (Midgley and Gray, 1971). Finally the act of transcription near the origin may in itself be the important factor in initiation and may be promoted by dnaA.

5.IV. DISCUSSION OF THE INITIATION PROCESS IN E.COLI

In summary, what are the events of initiation in E.coli? It is clear that there is some primary controlling process the nature of which is not certain. Following the controlling step there is a series of biochemical processes, most of which are defined primarily by the properties of mutant strains blocked at one of these subsequent steps. Finally, replication proceeds normally along the chromosome, initiation as such having been completed.

As discussed in section 5.II, it is not possible on present evidence to define the nature of the control mechanism. In a formal sense, the evidence presented in this thesis suggests that the F plasmid and the E.coli origin like other plasmids (see section 5.II) are able to maintain their concentration rather than their rate of replication. This probably means that initiation is negatively controlled as there are few well defined models involving positive control of replication and these do not predict that the copy number should be controlled, but rather that the rate of initiation should either be tied to some parameter of cell growth, or be constant. The repressor dilution model (Pritchard et al., 1969) as originally proposed links the production of repressor to the replication of a gene near to the origin, and thus it, like the unstable inhibitor model leads to the prediction that the origin concentration will be maintained. If it were proposed that inhibitor production is linked to initiation per se then the initiation frequency would be regulated.

The two inhibitor models of replication are reminiscent of a classical operon structure. Recently genetic evidence has been presented supporting such a system in the replication of ColE1. Shepard et al. (1979) have

reported that the deletion of a region of ColE1 adjoining the origin of replication results in a high copy number and that this high copy number phenotype is complemented in *trans* by another ColE1 plasmid or by a small self replicating fragment from the *ori* region of another ColE1 plasmid. Thus it seems that there is a negatively acting product involved in copy number control. Twigg and Sherratt (1980) have also isolated copy mutants of ColE1 in which the lesion maps near to the origin of replication. These mutations may be complemented by the ColK plasmid, but not by a colK plasmid having one restriction fragment deleted. It is interesting to note that ColK is compatible with ColE1 therefore they have presumably either not isolated true derepressed initiation mutants or incompatibility is not a direct reflection of the replication control mechanism. Further support for negative control of initiation may be drawn from the results of Gustafsson and Nordstrom (1978) who found an amber copy number mutant of plasmid R1*drd19*. Whilst it is conceivable that the mutant gene product could be a truncated initiator protein with an increased activity it is more likely to be a negatively acting protein which is inactivated in the mutant. Very recently Soll (1980) has isolated pseudovirulent mutants of a *λb221poriCasnA* transducing 'phage. These seem to carry *cis*-dominant copy number mutations in the *E.coli* origin fragment carried on the 'phage. One explanation of his results is that these are *oriC*^{c} mutants : in other words mutants in which the binding site for an inhibitor of replication no longer recognises the inhibitor. Ogura *et al*. (1980) found copy mutants of a mini F-*E.coli* minichromosome composite replicon. The mutations mapped on the *E.coli* fragment, 3kb from the *oriC* region proper near *unc*. Clearly these mutations cannot be complemented in *trans* as they were

isolated within an E.coli cell.

The weight of available evidence seems, therefore, to favour an inhibitor mechanism of initiation control which leads to maintenance of the origin concentration. It is interesting to note that Shuler et al. (1979) found that the inhibitor dilution model (Pritchard et al., 1969) was the only one out of those tested which yielded acceptable predictions concerning the timing of initiation and the lengths of the C and D periods when incorporated into a mathematical model for the growth of an E.coli B/r cell. Other initiation control models which linked the initiation to the concentration of precursors, the rate of envelope synthesis, the mass of cell envelope, or total cell mass were found to yield unreasonable predictions in limiting glucose concentrations. It is not possible, however to make a definite choice between such models at present, though the inhibitor dilution model seems a strong candidate.

Given that initiation is probably controlled by some inhibition mechanism that monitors the origin concentration, what is involved in the initiation process? It is clear that there are several steps in initiation. There is now good evidence that the dnaA gene product acts before that of dnaC. Zyskind et al. (1977) have demonstrated this by the analysis of the stages in initiation at which various antibiotics are inhibitory. Kung and Glaser (1978) have also demonstrated this by the use of dnaAts dnaCcs double mutants. It also seems that the dnaB gene product has an activity in initiation and that it acts before either of the other two (Zyskind and Smith, 1977; Lanka et al., 1978). Current evidence (Zyskind et al., 1977) suggests that dnaB product acts at a step in initiation involved in RNA synthesis since reinitiation upon a shift down is sensitive to rifampicin and streptolydigin. dnaA is involved at a subsequent step

which is sensitive to rifampicin but not streptolydigin and which therefore also requires RNA synthesis, while dnaC is involved in a still later step which is resistant to inhibitors of RNA and protein synthesis. All three steps seem to be independent of continued protein synthesis suggesting that there may be another step prior to the dnaB mediated step because initiation as a whole is sensitive to chloramphenicol (Maaløe and Hanawalt, 1961). Other gene products may well be involved in initiation. DNA gyrase seems to be involved in initiation (Fairweather et al., 1980; Filutowicz, 1980). There seems to be an interaction between gyrase and dnaA product (Orr et al., 1979; Filutowicz, 1980) as indicated by sensitivity of dnaA strains to inhibitors of the gyrB subunit and the difficulty of obtaining dnaA gyrB double mutants. Gyrase, or the gyrB subunit, also seems to be involved in some way in transcription (Fairweather et al., 1980) as indicated by the sensitivity of transcription to chlorobiocin - an inhibitor of the gyrB subunit. The exact role of gyrase remains obscure, although its effect on the degree of supercoiling of the chromosome (Gellert et al., 1976) might be expected to be pleiotropic. There is an interesting similarity between gyrase and dnaB product in that both are involved in initiation, and both have a role in continuing replication. This gives some support to the idea that there may be an initiation complex, at least some of the elements of which go on to play a part in ongoing replication as part of a replication complex. dnaB product has been found to have two enzyme activities associated with it (Lanka et al., 1978) but there is no evidence that these are connected with its two different roles.

Nusslein-Crystalla and Scheefers-Borchel (1979) have shown that the labelling of the immediate origin vicinity during initiation *in vitro* is anomalous and claim that this early region may be made by a special mechanism and that the replication forks proper start some distance from the origin on either side. This may fit with the finding of a delayed initiation in one direction relative to that in the other in plasmid R6K (Kolter and Helinski, 1978; Crosa *et al.*, 1978). Itoh and Tomizawa (1980) have identified an RNA primer in ColE1 which is thought to be replaced later by DNA.

What then is the nature of the first step? Protein synthesis and/or a structural change may be involved. Three possible roles for an inhibitor are (a) to act as a repressor in the synthesis of a positively acting factor (b) to protect the origin from a positively acting factor e.g. a nicking enzyme or (c) to hold the origin region in a certain structural configuration unfavourable for initiation. The last two are most in keeping with the proposition that stochastic effects govern the probability of initiation at the origin once inhibitor has diluted out whilst the first explains the known requirement for protein synthesis in initiation (Maaløe and Hanawalt, 1961). Evidence in favour of (c) is that often large numbers of direct and inverted repeats are seen when origins are sequenced, indicating that there may be considerable secondary structure in these regions. This feature has been seen in the origin regions of viruses, bacteriophages, plasmids large and small and of the bacteria. *E.coli* and *Salmonella typhimurium* (Soeda and Miura, 1979; Sims and Dressler, 1978; Denniston-Thompson *et al.*, 1977; Tomizawa *et al.*, 1977; Stuitje *et al.*, 1980; Stalker *et al.*, 1979; Meijer *et al.*, 1979; Sugimoto *et al.*,

1979; Zyskind and Smith, 1980). There may also be considerable conservation of this putative secondary structure between related replicons even though the primary structures may have diverged considerably (Zyskind and Smith, 1980; Stuitje et al., 1980). Although the secondary structures proposed are always speculative there usually seem to be "Pribnow Box" sequences in these regions of potential secondary structure, indicating the presence of RNA polymerase binding sites. This could possibly explain the need for DNA gyrase in initiation as it may be needed to produce the correct configuration for the binding of RNA polymerase or an RNA polymerase based initiation complex.

The relative ease of isolation of replication origins by recently developed techniques may aid in vitro studies of replication and lead to rapid advances in knowledge. Unfortunately control systems are notoriously difficult to study in vitro so that studies performed in vivo will remain vital to future advances in understanding.

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Chloramphenicol Releases a Block in Initiation of Chromosome Replication in a *dnaA* Strain of *Escherichia coli* K12

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Summary. DNA-DNA hybridisation experiments show that chloramphenicol induces a burst of initiation from the *oriC* region of a *dnaA46* mutant of *Escherichia coli* at 36.5° C but not from the isogenic *dnaA*$^+$ strain. Following this stimulation of initiation, DNA replication proceeds normally towards the terminus. The temporal pattern of the extra initiation is in parallel with the induced stimulation of RNA synthesis caused by chloramphenicol in the same strain. This is consistent with the hypothesis that the stimulation of initiation in the *dnaA* mutant is the result of the stimulation of the synthesis of an RNA species.

Introduction

When cultures of some temperature-sensitive *dnaA* mutants of *Escherichia coli* growing at partially restrictive temperatures are treated with chloramphenicol (CAM) there is a large and immediate increase in the rate of DNA synthesis (Messer et al., 1975). In such cultures, the amount of residual DNA synthesis is several times greater than that expected from the termination of existing rounds of replication, so it has been argued that CAM treatment causes, or enables, extra rounds of replication to be initiated in *dnaA* strains (Messer et al., 1975; Hansen and Rasmussen, 1977; Orr et al., 1978; Tippe-Schindler et al., 1979). This supposition has never been tested directly. In order, therefore, to see whether CAM induced stimulation of DNA synthesis is truly due to extra initiation we have investigated the pattern of chromosome replication by DNA-DNA hybridisation. The results show unequivocally that the addition of CAM to a strain carrying the *dnaA46* allele (Hirota et al., 1968), causes an immediate burst of initiation in the *oriC* region of the chromosome followed by replication of the remainder of the chromosome in the normal sequence (Bird et al., 1972). The pattern seen in a *dnaA*$^+$ strain is compatible with the termination of existing rounds of replication without further initiation.

In one *dnaA* strain it has been clearly documented that amino-acid starvation, in contrast to CAM, does not stimulate initiation (Orr et al., 1978). In order to account for this paradoxical observation it was suggested that it is not the inhibition of protein synthesis by CAM which is important in stimulating initiation. It was proposed instead that it is the stimulation of RNA synthesis, which is also induced by CAM (see Shen and Bremer, 1977) which is responsible. In support of this proposition it was shown that stimulation is also induced by amino-acid starvation if the *dnaA* strain is also *relA* (Orr et al., 1978). The various data reported here are all consistent with this hypothesis in that the kinetics of initiation after addition of CAM are similar to those found for stable RNA synthesis following CAM addition to bacterial cultures.

Materials and Methods

Strains. Bacterial strains and plasmids are listed in Table 1. RSF2124 has been described (So et al., 1975). pLG4 and pLG5 were isolated by R. Diaz, essentially according to Collins et al. (1976) by ligation of *Eco*R1-restricted RSF2124 DNA and *Eco*R1-restricted C600 DNA (R. Diaz, personal communication). pLG18 was isolated by G. Lycett in the same way except that W3110 DNA was used instead of C600 DNA and the plasmid was detected by screening a bank of Ap^R transformants by F′*lac* mediated transfer as described (Clark and Carbon, 1976). The fragment of chromosomal DNA in pLG4 is thought to be identical to that in pNK1 (Kozlov et al., 1977). λ318 was constructed by ligation of the *oriC* fragment of pLG6 (Diaz and Pritchard, 1978) into λNM781 (Murray et al., 1977). λ318 DNA was generously donated by W. Brammar.

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Table 1a. Strains

Strain	Genotype	Reference or origin	Source
A3	F⁻ *thr-1 leu-6 thi-1 lacY1 supE44 dnaA46* λ^- λ^s	Orr et al. (1978)	R.H. Pritchard
A3⁺	F⁻ *thr-1 leu-6 thi-1 lacY1 supE44* λ^- λ^s	Orr et al. (1978)	R.H. Pritchard
AB2463	F⁻ *thr-1 leu-6 thi-1 his-4 argE3 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 str-31 tsx-33 sup-37 recA13* λ^- λ^s	Bachmann (1972)	R. Diaz
LE496	AB2463 (pLG4)	See text	R. Diaz
LE497	AB2463 (pLG5)	See text	R. Diaz
GMS407 (CGSC5497)	F⁻ *argE3 lacY1 galK2 mtl-1 man-4 uidA 1 tsx-29 supE44?*	Novel and Novel (1973)	B. Bachmann
LE536	As GMS407 but *recA56*	Mating with JC5088 (Bachmann, 1972)	G. Lycett
LE541	LE536 (pLG18)	Transformation of LE536	G. Lycett

Table 1b. Plasmids and Phage

Plasmid	Vector	Molecular weight of chromosomal insert	Chromosomal markers and map location (min)		Source
pLG4	RSF2124	12.8 Md	*argE*⁺	88	R. Diaz
pLG5	RSF2124	7.5 Md	*proA*⁺	6	R. Diaz
pLG18	RSF2124	6.5 Md	*man*⁺	36	G. Lycett
λ318	λNM781	5.9 Md	*oriC*⁺ *asn*⁺	83	W. Brammar

Materials. Methyl-^{3}H-thymidine 5′ triphosphate was from the Radiochemical Centre, Amersham (40–60 Ci mmol^{-1}) or from I.C.N. (40–70 Ci mmol^{-1}). Methyl-^{3}H-thymidine was from Amersham. DNA polymerase grade 1 and proteinase K were from Boehringer Mannheim, and DNase I electrophoretically purified was from Worthington. All other biochemicals were from Sigma. Nitrocellulose filters for hybridisation were Sartorius 25 mm 0.45 μm pore size. Diphenylamine was recrystallised before use.

Isolation of Plasmid DNA. Plasmid-bearing strains were grown to A_{450} 0.5 and the plasmid content amplified for 15 h after the addition of 200 μg ml^{-1} CAM (Clewell, 1972).

Routinely a cleared lysate procedure was used which was based on that of Katz et al. (1973). Cells were washed and resuspended at a maximum concentration of 200 A_{450} units ml^{-1} in 25% sucrose 50 mM tris pH 8.0 on ice. For 3 ml of resuspended cells, 1 ml of a 4 mg ml^{-1} solution of lysozyme in 0.2 M tris pH 8.0 was added. After spheroplast formation, lysis was achieved by adding 1 ml 0.2 M EDTA pH 8 and after a further 10 min at 0° C, 5 ml of 2% Triton X100 in 50 mM tris 62.5 mM EDTA pH 8.0. The mixture was shaken well, left on ice for 20 min then centrifuged for 20 min, 40,000 g at 0° C and the supernatant saved. Strain LE541 would not produce good cleared lysates, so a "Hirt supernatant" was produced as described by Katz et al. (1977).

Both cleared lysates and supernatants were concentrated by precipitation with polyethyleneglycol 6000 (Humphreys et al., 1975) and the precipitate purified by banding twice in CsCl-Et Br gradients. The visible plasmid band was removed with a syringe, shaken 4 times with an equal volume of propan-2-ol, dialysed against 10 mM tris 1 mM EDTA pH 7.5 and stored at −80° C.

Isolation of Chromosomal DNA. 100 ml samples of culture were poured over 30 ml crushed M9 ice, 3 ml pyridine, centrifuged, washed, resuspended in 2 ml 10 mM tris 50 mM EDTA pH 8.0 and purified as described by Louarn et al. (1974) with the following modifications: the lysate was treated only once with pancreatic ribonuclease 1.25 mg ml^{-1} for 90 min at 37° C before treatment with proteinase K (250 μg ml^{-1} 120 min at 37° C). Pelleted cells were sometimes stored overnight at −20° C before lysis. The lysate was purified by equilibrium centrifugation in CsCl-EtBr. After taking fractions from the gradient the chromosomal band could be seen under longwave UV light and was treated as for plasmid DNA.

Labelling of Plasmid DNA. Plasmid DNA was labelled by nick translation. Volumes and concentrations were modified from those used by Macgregor and Mizuno (1976). The reaction mixture (1 ml) contained 50 mM tris pH 7.9, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg ml^{-1} bovine serum albumin, DNA (200 μg ml^{-1}) and dATP, dCTP, dGTP, dTTP (40 μM each). ^{3}H-thymidine deoxyribonucleoside triphosphate 240 μCi in 50% ethanol was evaporated to dryness in a dessicator, the above mixture added to it and the mixture preincubated at 15° C for 15 min. The reaction was started by adding DNase (10^{-5} mg) and DNA polymerase (30 units). The mixture was incubated 75 min at 15° C and the reaction stopped by the addition of 50 μl 20% SDS and heating to 60° C for 3 min. Samples were taken for the determination of percentage incorporation of label into acid precipitable material. The mixture was extracted three times with equal volumes of phenol, dialysed extensively against 10 mM tris 1 mM EDTA pH 7.5 and stored at −80° C. Tests showed that the DNA concentration could be varied from 200 μg ml^{-1} to 25 μg ml^{-1} in which case the triphosphate concentrations were scaled down in proportion. This method routinely gave 15–55% incorporation of label, the optimum incubation time being 1–1$^1/_2$ h. The resulting DNA gave a normal restriction pattern and was suitable for DNA-DNA hybridisation. We found $1.0–5.0 \times 10^5$ counts min^{-1} to be a sufficiently high specific activity though much higher levels could theoretically be obtained by using a higher specific activity of dTTP.

DNA-DNA Hybridisation. Hybridisation techniques were as described by Louarn et al. (1974) except that incubation was at 45° C for 4 days and 2 washes in 2 × SSC 50% formamide and 3 washes in 2 × SSC were performed at 40° C. It was found that these modifications significantly reduced the level of non-specific binding of label to filters. Filters were baked at 80° C for 2 h. Vacuum was found to be unnecessary. Filters were loaded with 2 μg *E. coli* DNA and hybridised to nick translated plasmid DNA as probe. Unloaded filters were similarly treated and the counts subtracted from corresponding values for loaded filters. Probe DNA was sonicated in an MSE 150 watt ultrasonic disintegrator at 15–18 μm amplitude with a 3 mm exponential probe.

Measurements of DNA and Cell Mass. Cell mass was measured as A_{450} in a Gilford microsample spectrophotometer. For estimation of DNA content, samples of culture (50 ml) were taken into an equal volume of ice-cold 15% trichloroacetic acid and kept at 0° C for at least 90 min after which they were filtered onto 4.7 cm membrane filters. The filters were allowed to dry, immersed in 0.5 M perchloric acid and heated at 70° C for 30 min. The liquor was immediately decanted. DNA concentration was measured by the method of Burton (1956) except that the acetaldehyde was added last and the colour was measured as the difference between A_{595} and A_{700} as recommended by Giles and Myers (1965).

The concentration of DNA purified for hybridisation was measured spectrophotometrically at 260 nm.

Pulse Labelling. 0.5 ml duplicate samples of culture were pulsed for 2 min using 2 μCi ml^{-1} ^{3}H-thymidine (85 Ci $mmol^{-1}$) or 4 μCi ml^{-1} (2 μg ml^{-1}) ^{3}H-uridine. The pulse was stopped with 2 ml 10% trichloroacetic acid. Samples were left for 30 min, washed with cold 5% trichloroacetic acid and 90° C distilled water before counting.

Results

We have confirmed by pulse labelling (Fig. 1a) that there is a stimulation in the rate of DNA synthesis when strain A3, grown at 36.5° C, is treated with CAM. In our experiment the rate of incorporation reached a maximum, of about six times the pretreatment rate, after 40 min. In the *dnaA*$^+$ control culture the rate of DNA synthesis fell gradually after the addition of CAM. These results agree with those published previously (Messer et al., 1975; Tippe-Schindler et al., 1979). Since pulse labelling data may be affected by changes in precursor pool sizes, we also made direct chemical measurements of the DNA content of the cultures after the addition of CAM (Fig. 2). These measurements show that DNA was synthesised exponentially for more than 30 min, with a doubling time of 21 min. Since strain A3 normally doubles in mass in 44 min in the conditions used, this represents a doubling in the exponential rate of DNA synthesis. The rate of DNA synthesis between points on Fig. 2 has been estimated and the estimations included in Fig. 1a for comparison. It may be seen that the rates of DNA synthesis as estimated from pulse labelling and from chemical measurement of DNA are in good agreement. Measurements of the rate of RNA synthesis by pulse labelling after the addition of CAM to strain A3 (Fig. 1b) show

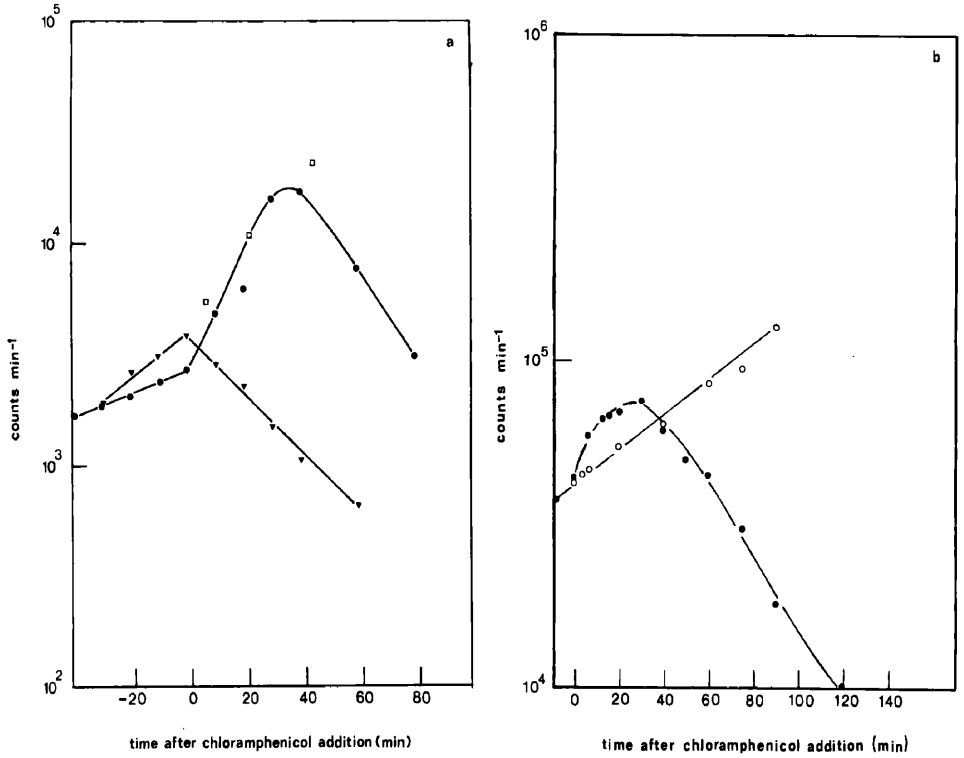

Fig. 1a and b. Pulse labelling was performed as described. Samples were taken from the cultures described in Fig. 3. ● A3; ▼ A3$^+$; o A3 control without CAM; □ represents points calculated from the data for A3 in Fig. 2. Points represent the mean change in DNA content over the period between 2 points in Fig. 2. The figures were normalised against the pulse labelling data assuming that the rate of DNA synthesis at 0 min = growth rate of culture. **a** DNA synthesis; **b** RNA synthesis

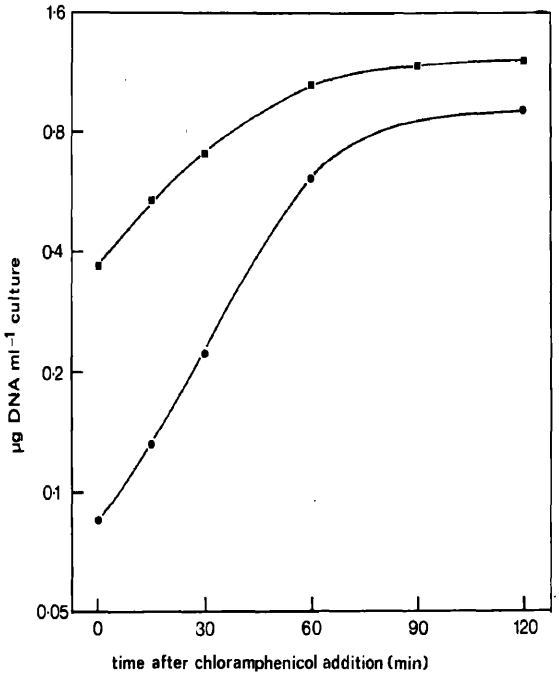

Fig. 2. Cultures were grown at 36.5° C and CAM added at A_{450} 0.2 (time 0). Samples were taken at times indicated and DNA estimated as described. ● A3; ■ $A3^+$

a pattern very like that for DNA synthesis except that the stimulation is slightly shorter lived. Thus the pattern of RNA synthesis seems to be following the pattern of initiation of chromosome replication rather than that of DNA synthesis per se. This is in line with prediction (Orr et al., 1978). The data also agree with published data for RNA accumulation after CAM addition (Midgley and Gray, 1971).

Using the cultures from which the samples for pulse labelling were taken, samples were removed and the DNA purified for hybridisation in order to deduce the pattern of replication of the chromosome. The results of the hybridisation are shown in Figs. 3a and 3b for strain A3, and for strain $A3^+$ in Fig. 3c. The change in marker ratios was estimated as explained in the figure legend. A sudden burst of initiation at the normal origin of replication would be expected to give an immediate rise in the ratio of *oriC* to the other markers, and this is seen clearly as a consequence of the addition of CAM to strain A3 (Fig. 3a). Furthermore, if this burst of synchronous initiation were to give rise to a synchronous wave of replication forks, it would be expected that the ratio of each marker to markers farther from the origin would rise discretely as the wave of replication forks passed through that part of the chromosome. We saw just such a pattern in strain A3 (Fig. 3a). The times at which the marker ratios changed are consistent with the normal pattern of replication (Bird et al., 1972) and a replication time of 40–50 min.

From the data in Fig. 2, which show a doubling in the rate of DNA synthesis within a few minutes, we would expect a doubling in the number of replication forks soon after the addition of CAM, since the doubling time of strain A3 before the addition of CAM was 44 min. Assuming that the normal replication time is also ≃45 min there will be on average one replication fork per chromosome terminus and an origin:terminus ratio of 2:1. A doubling in the rate of DNA synthesis at constant replication fork velocity would reflect a doubling in the number of replication forks and a rise of 50% in the origin:terminus ratio to 3:1. The rise of 20%–40% seen in the *oriC*:*man* and *argE*:*man* ratios after 15–30 min is not inconsistent with this prediction since the hybridisation figures underestimate the change in marker ratio due to uncorrected "nonspecific hybridisation" as explained in the legend to Fig. 3.

If, as we believe, ongoing replication continues at a normal rate in strain A3 after the addition of CAM, termination of rounds of replication will continue to occur for some time at the pretreatment rate. The continued rise in the ratio of origin markers:terminal marker must therefore indicate that initiation continued, at a level higher than before the treatment, for at least 30 min. These findings account for the nearly exponential rate of DNA synthesis seen in Fig. 2, since the number of chromosomes was still increasing at the old exponential rate and at the same time the number of replication forks per chromosome rose to nearly twice the pretreatment level, and possibly continued to rise for more than 30 min.

The marker ratios 90 min after treatment are difficult to interpret in detail since all rounds of replication initiated before addition of CAM would have completed. Nevertheless it is clear that the rate of initiation must by this time have fallen at least to a level below the pretreatment level and possibly to zero, though all rounds of replication may not have terminated.

Figure 3c shows that the pattern of change in the marker ratios of the $dnaA^+$ control is consistent with the cessation of initiation and the completion of existing rounds of replication.

The results also show that the total quantity of residual DNA synthesised is not greater than can be accounted for by a return to the DNA:mass ratio found in $dnaA^+$ cultures. Comparing the data for strains A3 and $A3^+$ in Fig. 2 it may be seen that before the addition of CAM, the exponentially growing cultures had a 4.4 fold difference in DNA:mass ratio at 36.5° C. This is in good agreement with published data (Orr et al., 1978; Pritchard et al., 1978). 120 min after treatment when DNA synthesis had almost ceased, the difference was reduced to 1.35-fold.

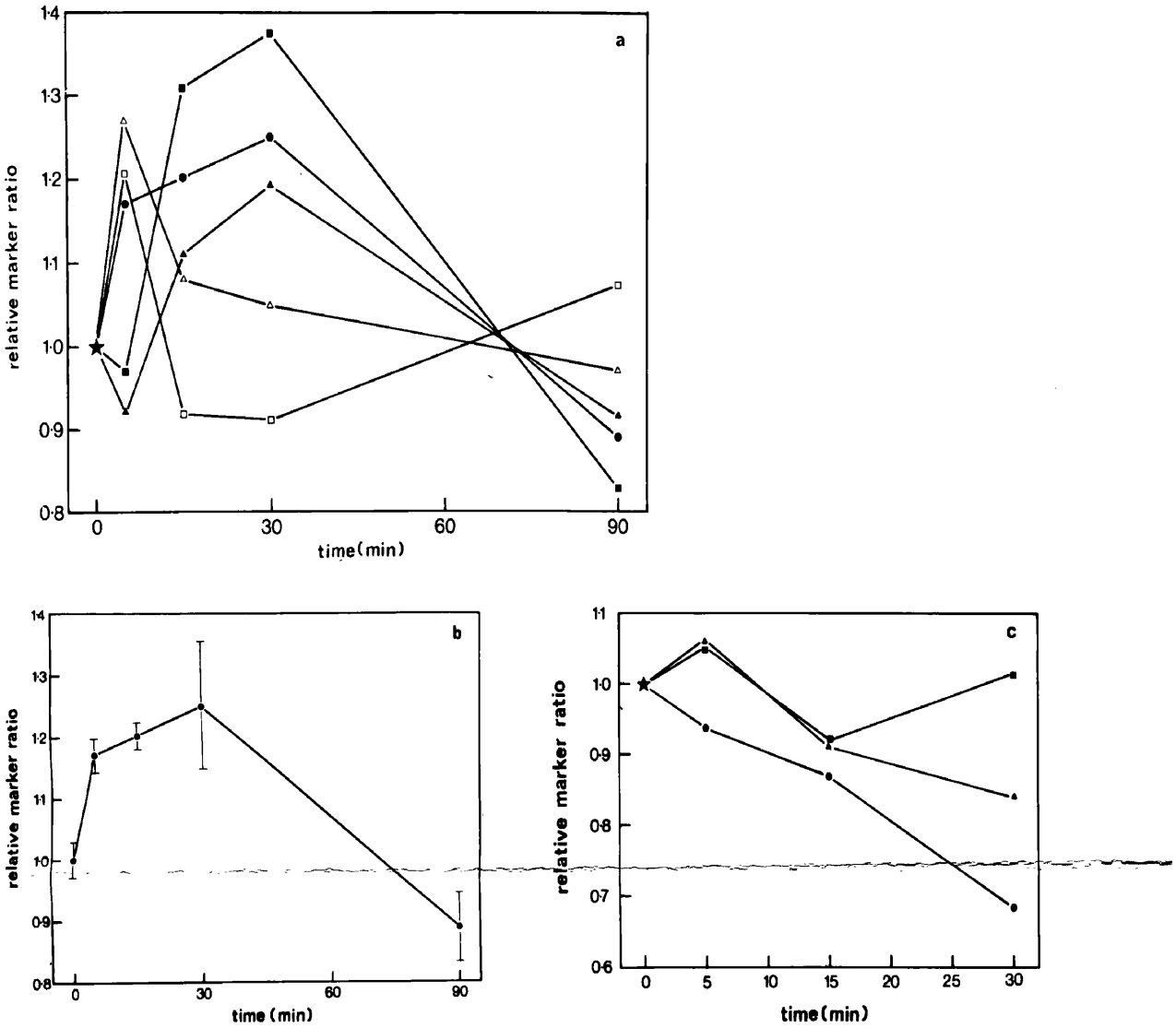

Fig. 3. a A culture of A3 was grown for 5 generations at 36.5° C and CAM (150 μg ml^{-1}) was added at A_{450} 0.2 (time 0). Samples were taken at times −20, 0, 5, 15, 30 and 90 min and purified as described under materials and methods. Twelve filters were loaded with each sample of DNA and three hybridised to each of the labelled probes, λ318, pLG4, pLG5, pLG18. 12 blank filters were similarly treated and the mean number of counts binding to the blank filters for each probe were subtracted from the corresponding values for the loaded filters. The ratio of counts binding for any samples when hybridised to each pair of probes was calculated. The ratios were then normalised against the corresponding ratio for the mean of the two pretreatment samples. The number of counts binding to the filters are uncorrected for "nonspecific hybridisation", thus the change in ratios of counts underestimates the true change in relative gene dosage. We have estimated this nonspecific hybridisation to represent not more than 15–20% of the total counts when pLG4 is used as a probe (data not shown). ● *oriC:man*; ■ *argE:man*; ▲ *proA:man*; □ *oriC:argE*; △ *oriC:proA*. b The error bars represent the standard error for each point. The standard errors shown for the *oriC:man* ratios are fairly representative of those found for other marker ratios. Key as for Fig. 3a. c A culture of $A3^+$ was similarly treated as A3 (see Fig. 3a) except that no 90 min sample was taken. Data were treated as in Fig. 3a. Key as for Fig. 3a

This implies that the DNA:mass ratios of the two cultures were approaching the same value.

Discussion

Our method of DNA-DNA hybridisation, which employs suitable probes chosen from a collection of cloned fragments of *E. coli* DNA, offers a direct and easy method of investigation of the pattern of chromosome replication, without the need for laborious construction of multiply lysogenic strains.

We have shown unambiguously that the stimulation of DNA synthesis in a *dnaA46* strain upon the addition of CAM is due to extra initiation in the region of the chromosome origin *oriC*. The data also suggest that the cycles of replication initiated under this treatment proceed normally to termination. This

supports the commonly accepted assumption that this type of replication is not aberrant in form, and that only the replication control mechanisms are affected in *dnaA* strains. The mechanism by which the control is affected is less clear. Several possibilities have been entertained (Hansen and Rasmussen, 1977; Orr et al., 1978; Tippe-Schindler et al., 1979). All are based on the assumption that initiation induced by CAM is related to the delay in initiation during exponential growth of *dnaA* strains at intermediate temperatures. That such a delay exists is shown by the fact that although the rate of replication fork movement is not materially altered under these conditions (Orr et al., 1978) the DNA concentration (DNA:mass ratio in an exponential culture) is lower than in corresponding $dnaA^+$ strains and varies inversely with the incubation temperature (Hansen and Rasmussen, 1977; Fralick, 1978; Pritchard et al., 1978; Orr et al., 1978). There is thus a deficit of DNA in such cultures. In addition it has been shown that at temperatures up to 37° C the amount of initiation induced by CAM is positively correlated with the incubation temperature before CAM addition, and hence with this deficit in DNA caused by the *dnaA* mutation (Hansen and Rasmussen, 1977).

In other words it is assumed that *dnaA* mutants acquire a 'capacity' for initiation which can be expressed by addition of CAM to the culture. In this respect CAM suppresses the DnaA phenotype. It is the proposed nature of this 'capacity' and the role of the *dnaA* product which distinguishes the models of the three groups referred to above.

Orr et al. (1978) propose that the primary control step in initiation is normal in *dnaA* strains and that the $dnaA^+$ gene product is involved in a later step which requires the synthesis of an RNA. There is other evidence for such a proposal (Zyskind and Smith, 1977; Zyskind et al., 1977). This later step is not the rate limiting (or controlling) one in initiation of wild-type cells during steady state exponential growth, but becomes the rate limiting one in *dnaA* strains because of the reduced activity of the *dnaA* gene product. Capacity, then, is the accumulation of origins in the culture in which the first step in initiation has already taken place but which are held up at this transcriptional, or RNA-mediated step. Addition of CAM, by stimulating stable RNA synthesis, increases the probability that this step will be accomplished by a mass action effect.

Hansen and Rasmussen (1977) assume that the $dnaA^+$ gene specifies a positively acting protein which determines the frequency of initiation during exponential growth of wild-type cells and is "used up" in stoichiometric amounts in this process. It is thus the primary control gene. In *dnaA* mutants there is less active *dnaA* product, but over-production of an inactive form as a result of auto-regulation. It is presumably this inactive form which represents capacity and ultimately expresses itself by renaturing (or becoming active) in the presence of CAM.

There are several fundamental difficulties with this hypothesis. Why is capacity not expressed during amino-acid starvation? Why does inhibiting protein synthesis with CAM cause a *stimulation* in the rate of initiation when there is no change in the temperature to alter the renaturation kinetics of the monomer? Is it conceivable that the primary initiation control – which ensures a precise timing of chromosome initiation in the cell cycle (Koppes et al., 1978) would also be obligatorily required for the replication of plasmids such as pSC101 (Hasunuma and Sekiguchi, 1977; 1979; Frey et al., 1979) and probably of F (Tresguerres et al., 1975), which do not replicate in synchrony with the chromosome? If $dnaA^+$ product were used up stoichiometrically in initiation would not the presence of such plasmids have serious quantitative effects on chromosome initiation?

The data of Hansen and Rasmussen (1977) show that when expressed at the growth temperature, capacity was sufficient to make up the acquired deficit in DNA concentration as reported here. However, when expressed at 28° C, capacity was greater than this. This result is consistent with the model of Orr et al. (1978) if it is assumed that the step in the initiation process at which the *dnaA* gene product is involved occurs before the step responsible for the production of a stable inhibitor (Pritchard et al., 1969). In this case, the normal control circuit will not have acted before the addition of CAM and will be inhibited by CAM. Since the data presented here show that the kinetics of RNA synthesis after the addition of CAM agree well with the kinetics of initiation, we would expect initiation to be limited only by the degree of CAM-induced stimulation of RNA synthesis and the proportion of *dnaA* product in the active state.

Tippe-Schindler et al. (1979) propose a third explanation for capacity which predicts a stimulation of DNA synthesis following amino acid starvation as well as following the addition of CAM. These authors claim that amino acid starvation stimulates initiation in a strain carrying one *dnaA* mutation (*dnaA5*) Orr et al. (1978) found no such stimulation with *dnaA46*, nor have we observed such an effect with *dnaA5* in a K12 background (to be published). These differences may be a consequence of the genetic background and in particular, a consequence of the effect of this background on RNA synthesis under conditions of amino acid starvation.

What role does the *dnaA* product play in initia-

tion? Several pieces of evidence implicate it as being involved in the synthesis of an RNA species catalysed by RNA polymerase. Bagdasarian et al. (1977) have found that some suppressors of *dnaA* map in the *rpoB* gene specifying a subunit of this enzyme. Orr et al. (1978) have shown that *dnaA* mutants are exceptionally rifampicin sensitive and that the suppression of *dnaA* by CAM is a relaxation-like effect. In addition, of course, we know that RNA polymerase is required for initiation because initiation is rifampicin sensitive (Lark, 1972) and because a thermosensitive copy mutant of *E. coli* maps in the *rpoC* gene (K.V. Rasmussen, personal communication). Among the suggestions being widely canvassed are: that the $dnaA^+$ gene product is a ρ-like protein which terminates the synthesis of a unique RNA species synthesised at the chromosome origin (Atlung, 1979); or that it is a λN-like protein which acts as an antitermination protein promoting the synthesis of such an RNA species (Bagdasarian et al., 1978). The $dnaA^+$ product could also be like Tu-Ts factor (Travers et al., 1970) in that it may make RNA polymerase preferentially transcribe one class of RNA (in this case an initiation specific RNA).

A rather different type of suggestion follows on from the model of Orr et al. (1978). The $dnaA^+$ product may have a specific RNA as a substrate, or the origin region of the chromosome may be conformationally altered by transcription such that it becomes a substrate for $dnaA^+$ product.

There is little evidence at present to permit a choice between these alternatives.

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ABSTRACT

THE CONTROL OF INITIATION OF DNA REPLICATION IN ESCHERICHIA COLI

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A new type of DNA-DNA hybridisation system was used to investigate two aspects of the initiation of replication in E.coli.

A colony bank of recombinant plasmids carrying various fragments of the E.coli chromosome was constructed by manipulation in vitro. A number of plasmids carrying specific E.coli genes were selected by direct complementation analysis or by F'lac mediated mobilisation. These plasmids together with other similar ones were used as hybridisation probes. The conditions for hybridisation and the labelling of DNA in vitro were refined.

DNA-DNA hybridisation and chemical DNA measurements were used to demonstrate that chloramphenicol induces a burst of extra initiation at the origin of a dnaA46 strain growing at a semi-permissive temperature. The temporal pattern of the initiation is consistent with the hypothesis that the stimulation of initiation is the result of the stimulation of the synthesis of an RNA species.

It was shown that there is a disturbance in the pattern of replication of Hfr strain KL99 during exponential growth compared with that in a closely related F^- strain. The pattern is consistent with initiation occurring at the F origin. The DNA/mass ratio of Hfr AB313 is not changed relative to that of the isogenic F^- strain, indicating that the F plasmid origin of replication is not active in this strain during exponential growth. As both Hfr strains are capable of giving rise to F' plasmids it is argued that the difference in the point of insertion of F in the chromosome is the cause of the difference in the initiation pattern between the two strains. These results, are interpreted as being a reflection of a copy number titration control mechanism for F replication.

Possible mechanisms for the primary control and for the subsequent steps in the initiation process are discussed.