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Identification of gene products programmed by restriction endonuclease DNA fragments using an E. coli in vitro system

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ABSTRACT

DNA restriction enzyme fragments have been used to programme the synthesis of polypeptides in an in vitro system without apparent loss in fidelity compared with supercoiled templates. The system is extremely sensitive, less than 1 μg of DNA can be used to direct the synthesis of ^{35}S -labelled polypeptides of sufficiently high specific activity such that products can be identified by SDS-PAGE after a few hours autoradiography. The ability to analyse fragments can be used to readily assign specific proteins to small regions of the coding template, to identify cloned gene products distinct from those of the vector, and to identify cloned genes expressed from their own promoters. The in vitro system can be used successfully with bacterial DNA from other species and efficient extracts can be prepared from any E. coli K-12 strain, which should greatly facilitate the purification of factors controlling the expression of specific genes by complementation assay.

INTRODUCTION

A major requirement in gene cloning studies is a rapid and reliable system for the identification of gene products, particularly if these are coded by previously uncharacterised genes. Such products can be identified by infection of UV-irradiated lysogenic hosts with phage vectors carrying foreign DNA (hybrid phages), UV-irradiation of host cells carrying high copy number, hybrid plasmids (maxi-cell system) or anucleate mini-cells, either containing hybrid plasmids or infected with λ carrying cloned sequences. These systems are limited to the use of specialised transducing phages such as λ, and to multicopy plasmids used in conjunction with special mutant strains of E. coli producing mini-cells. Moreover, the specific matching of a cloned gene

with a previously unidentified polypeptide product, and whether that gene has been cloned together with its own promoter, is frequently difficult to establish using *in vivo* systems. In an attempt to circumvent these problems and in order to develop a convenient routine system for gene product identification we have re-investigated the use of the *in vitro* coupled transcription/translation system based on that originally described by Zubay (1) and modified by others (see 2). In particular, we have examined the ability of restriction endonuclease digests of DNA from λ or plasmid clones, or purified restriction fragments, to direct the synthesis of a wide variety of polypeptides *in vitro*. Konings *et al* (3) and Lindhal *et al* (4) previously demonstrated that linear DNA templates could be used to direct polypeptide synthesis in an *in vitro* system and were able to map the position of a number of M13 phage proteins and several ribosomal protein genes from *E. coli* respectively. These analyses however were mostly limited to small polypeptides and the *in vitro* system used involved the reconstitution of a number of separately purified components. The procedure is therefore relatively laborious and has not been generally adopted. In contrast, the system we describe here is remarkably simple to use.

Since we wanted to use DNA fragments to programme the *in vitro* system we first examined the effect of supercoiling on gene activity and conclude that it is not a major requirement for the expression of genes *in vitro*. Although, in some cases, the overall efficiency of protein synthesis from linear molecules was reduced 3-4 fold compared to the equivalent supercoiled template, the fidelity of the products was nevertheless preserved and the level of synthesis achieved was still sufficient to require only a few days autoradiography to complete the analysis. The use of small defined DNA fragments as coding templates allows the assignment of polypeptides and their corresponding promoters to specific regions of cloned DNA. Moreover, the ability to analyse separately passenger and vehicle DNA also readily identifies any fusion polypeptides (which might otherwise confuse the analysis) coded across the boundaries of the insert. The enormous resolving power of the *in vitro* system is equally matched by its convenience and simplicity of use particularly

since we now find that active extracts can be prepared routinely from several E. coli K12 (RNase$^+$) strains. This finding opens up the possibility for more extensive in vitro studies of gene regulation by using extracts from mutants altered in the control of gene expression. Finally, preliminary results indicate that the system can be used to read DNA templates from a wide range of bacteria.

MATERIALS AND METHODS

Bacterial Strains. The following E. coli strains were used: DS410, minA, minB (obtained from Professor D. Sherratt); PAM 162, thr, leu, his, met, thi, lon, sulB26 (B. Bachmann) served as host for pLG510; MRE600, RNase I$^-$, was used as source of the S30 extract for in vitro protein synthesis; LE234, metB, argE, ilv, tna, supE and its gyrB mutant have been described previously (5); Staphylococcus aureus was used as the source of plasmids pC221 , pCW41 and pCW46 all carrying Cm^R (obtained from Professor W. Shaw). Hosts for all other plasmids were standard laboratory E. coli K12 strains.

Plasmids. The following plasmids were used, Col E1; pBR325 (Ap^r, Cm^R, Tc^R) and its derivative pLG282 carrying a single EcoRI fragment and api$^+$ (6); pLG215 also a derivative of pBR325 carrying a single EcoRI fragment coding sog$^+$ (7); pKN410, Ap^R (obtained from K. Nordstrom) and its derivative pLG510, carrying a single EcoRI fragment coding envA$^+$ (8); pLG310 a pSF2124 derivative carrying a single EcoRI fragment coding for dacC (penicillin binding protein 6) (9).

Purification of DNA fragments. Restriction enzyme fragments were separated in preparative gels ranging from 0.3 to 1% $^w/_v$ as appropriate. DNA bands were cut out of the gel, placed in a dialysis bag and separated from the agarose by electrophoresis. The DNA was then concentrated by treatment with isobutanol and precipitated with ethanol.

Linearising of circular DNA molecules. Plasmid DNA was cleaved with appropriate restriction endonucleases under standard conditions. Reactions were checked for completion by running DNA samples in agarose gels. The DNA was then precipitated with ethanol, pelleted, dried and resuspended in 1mM Tris,

0.1mM EDTA pH 7.5.

Preparation of plasmid DNA. Plasmid DNA for use in vitro was prepared and purified by centrifugation on CsCl-ethidium bromide gradients essentially as described by Davis & Vapnek (10).

Relaxation of supercoiled DNA. Topoisomerase I was purified according to the method of Depew et al (11) incubated with supercoiled plasmid DNA and checked for complete conversion to the relaxed form by agarose gel electrophoresis as described by Orr and Staudenbauer (12). The DNA was finally recovered by ethanol precipitation as above.

Gel electrophoresis. Radioactive samples were analysed in 11 or 15% $^{w}/v$ polyacrylamide gels and the gels finally autoradiographed as described previously (13).

Synthesis of polypeptides in mini-cells. The mini-cell producing strain DS410 was transformed with plasmid DNA essentially as described by Dagert & Ehrlich (14) with selection for appropriate antibiotic resistance. Mini-cells were then purified as described (15) and incubated with ^{35}S -methionine (25μCi; 50μCi/μg) for 30 min at 37°C, mixed with an equal volume of electrophoresis sample buffer and analysed by SDS-PAGE.

Synthesis of polypeptides in vitro. The S30 extract used to promote coupled transcription/translation in vitro was prepared from MRE600 essentially as described by Collins (2). The extract was stored in liquid nitrogen and could be thawed and re-frozen once without loss of activity. The standard reaction mixture (30μl) usually contained 1.5 to 2.5μg of DNA in 5μl; 5μl S30 extract; 2μl ^{35}S -methionine (7μCi/μl) ; 1 to 3μl Mg acetate (0.1M); 7.5μl low molecular weight mix as detailed by Collins (2) and 1mM Tris-acetate, pH7.0. Protein concentration of the S30 was usually about 100 mg/ml but this may vary between 30 and 200 mg/ml with little effect on efficiency. For each preparation the optimum Mg^{++} concentration was experimentally determined. The reaction was started by adding the S30 extract and incubating in an eppendorf tube at 37°C for 30 to 60 min with gentle shaking; 5μl of prewarmed methionine (44mg/ml) was then added and incubation continued for a further 5 min. The reaction was stopped by placing on ice; a 2μl aliquot was removed, precipitated

with ice cold 5% $^{w}/_{v}$ trichloracetic acid (TCA) and counted; the remaining sample was immediately mixed with an equal volume of electrophoresis sample buffer (containing 4% $^{w}/_{v}$ sodium dodecyl sulphate), boiled for 5 min and stored at -20^{o}C until analysed by SDS PAGE. A maximum of 15µl of the original reaction mixture may be applied per gel slot without overloading with protein (usually a minimum of 5 x 10^{5} cpm was loaded per slot). The dried down gels were then autoradiographed overnight or for a few days as appropriate. Extreme care was taken throughout the procedures outlined above to maintain sterility and to avoid contamination with RNase. The use of RNase in the purification of DNA should be specifically avoided since even repeated phenol extraction does not remove all the RNase activity from the DNA. In addition, DNA should be free of CsCl, EtBr and high salt if efficient template activity is to be achieved.

RESULTS

Characteristics of the *in vitro* transcription-translation system adopted

The basic system used in this study was virtually identical to that described by Collins (2) which includes a few modifications of the original Zubay (1) procedure. The main modifications are the avoidance of high concentrations of Cl^{-} ions, which have been reported to inhibit *in vitro* transcription (16), the omission of co-factors FAD and PAB and the treatment of water and glassware used in the preparation of the S-30 extract and LMM (low molecular weight mix) components with the nuclease inhibitor diethyl pyrocarbonate. In addition, we found that low speed centrifugation of the S-30 extract, immediately before use, removed any remaining whole cells and large debris and reduced the level of background incorporation. To achieve maximum synthesis it was useful to optimise the DNA concentration with the standard S-30 concentration. The S-30 preparation contains considerable amounts of cellular membrane and we have observed that the additon of inner membrane vesicles to the incubations reduces protein synthesis suggesting that membrane may be inhibitory. This effect can be titrated out by increasing the

amount of added DNA or more economically by reducing the S-30 concentration.

[^{35}S]-methionine was used both as label and as the sole added source of methionine for protein synthesis,which continued at a linear rate for at least 30 min, when programmed with up to 5µg of supercoiled plasmid DNA. In comparing the *in vitro* and minicell (*in vivo*) systems it is important to note that, due to the ability to label at extremely high specific activities *in vitro*, the efficiency of labelling with [^{35}S]-methionine is substantially greater using the *in vitro* system. Using the mini cell procedure as described by Dougan and Sherratt (15) the required exposure times for autoradiographs or fluorographs are frequently 10-15 fold longer than for the equivalent *in vitro* analysis despite the addition in some cases of 5-fold more [^{35}S]-methionine to the minicells.

Three minor polypeptides, molecular weights 60K, 18K and 16K were frequently detected in samples from the *in vitro* system incubated in the absence of added DNA. In the case of the 60K protein its formation disappears with length of storage of the S-30 and in addition is strain dependent; Zubay extracts prepared from K12 strains produced very little of this polypeptide

Effect of DNA gyrase on gene expression *in vitro*

Yang *et al* (17) reported that the level of expression of certain genes (e.g. Colicin E1) in an *in vitro* transcription/ translation system was reduced by inhibitors of DNA gyrase, although in at least some cases they found that supercoiled and relaxed DNA molecules were equally efficient as templates for these genes. In our studies, clorobiocin, an inhibitor of the *gyrB* subunit (18) had no effect on DNA dependent protein synthesis *in vitro* when added to a wild type *gyrB*$^{+}$ extract. Moreover, an extract prepared from *E. coli* LE316, a mutant which completely lacks *gyrB* activity *in vitro* (5) was just as efficient in promoting protein synthesis directed by the ColE1 plasmid, pLG310, as the wild type extract. In our experience the degree of supercoiling of DNA, whether added in supercoiled or relaxed form to the *in vitro* extract, is probably determined by the relative activities of gyrase and the relaxing enzyme topoisomerase I. We therefore carried out additional control

experiments, in which protein synthesis directed by wild type or gyrB extracts, were compared when programmed with ColE1 DNA in either supercoiled or relaxed (by prior treatment with topoisomerase I) form. Under these conditions we were able to demonstrate that the relaxed molecules were rapidly supercoiled in the wild type extract, whilst they remained relaxed and covalently closed in the gyrB extract (data not shown). Nevertheless, the overall efficiency of the relaxed ColE1 template in the absence of gyrase activity was only reduced by 30% and in fact using pBR325 DNA as template the overall incorporation actually increased by 60%. Finally, analysis of the products formed by relaxed and supercoiled templates, shown in Fig. 1 indicated no significant differences. We conclude therefore that under the conditions of this in vitro system supercoiling of DNA templates is not a major limiting factor.

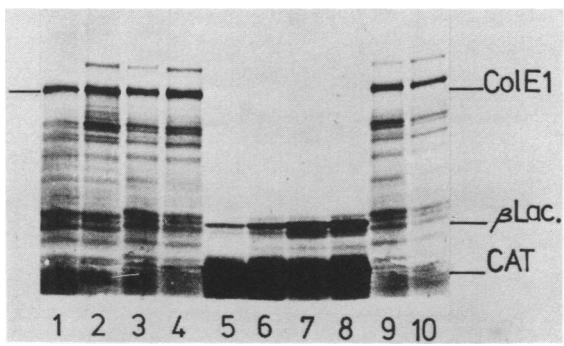

Figure 1. Synthesis of proteins in vitro from supercoiled and relaxed plasmid DNA templates. 2µg of DNA was used to programme the in vitro system in the presence of ^{35}S - methionine and the polypeptide products were analysed by SDS-PAGE followed by autoradiography. The S30 extracts used were prepared from LE234 (wild type), tracks 1,2,5,6 or from the gyrB mutant LE316, tracks 3,4,7,8,9,10 (9 and 10 contain 1mM banzamidine). Track 1, supercoiled ColE1; 2, relaxed ColE1 (with topoisomerase I) and in the presence of 25µg/ml clorobiocin; 3, supercoiled ColE1; 4, relaxed ColE1; 5, supercoiled pBR325; 6, relaxed pBR325 plus clorobiocin; 7, supercoiled pBR325; 8, relaxed pBR325; 9, supercoiled ColE1; 10, relaxed ColE1. The position of the β-lactamase and CAT proteins programmed by pBR325 and the Colicin E1 protein are indicated.

Synthesis of polypeptides from linear and supercoiled templates and the identification of fusion proteins generated at ligation sites

Since we have shown that supercoiling is not absolutely necessary for efficient transcription we proceeded to investigate the ability of restriction mixtures and purified restriction fragments to direct synthesis in vitro.

Plasmid pLG310 codes for β-lactamase and the E. coli penicillin binding protein, PBP6. The template capacity of this supercoiled plasmid DNA was compared with that of the fragments generated by EcoRI digestion. The results obtained are shown in Fig. 2a. Tracks 1 and 2 demonstrate that linearising the

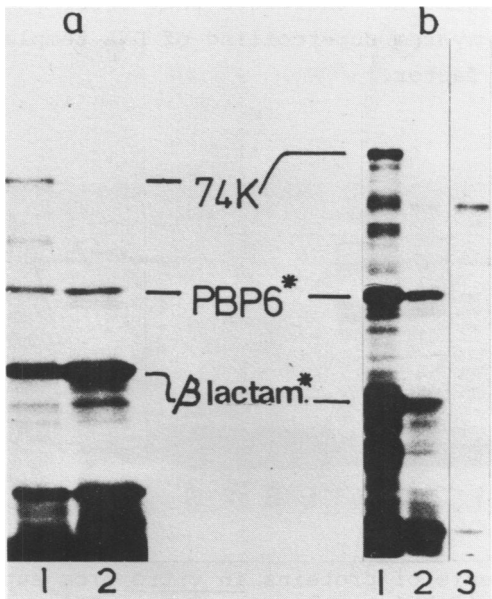

Figure 2. Polypeptide synthesis from supercoiled and from linear DNA templates. Equimolar amounts of DNA were used in separate reactions using an S30 prepared from MRE 600. Gel a, track 1, supercoiled pLG310; 2, pLG310 completely cleaved with EcoRI. Gel b, track 1, pLG310; 2, 5.85kb linear EcoRI fragment from pLG310 coding for dacC (PBP6); 3, no DNA. Samples in Gel b exposed for a longer period. In the in vitro system both PBP6 and β-lactamase are synthesised as larger molecular weight precursors, PBP6* and β-lactamase* (19). Note the complete disappearance of the 74K protein when pLG310 is cut with EcoRI.

template had no effect on the expression of β-lactamase, PBP6 and several unidentified polypeptides. Comparison of tracks 1 and 2 also shows that a 74K polypeptide coded by the intact pLG310 molecule was completely abolished by cleavage with EcoRI. This protein is not programmed by the (supercoiled) parent vector, ColE1 (not shown) or the purified 5.85Kb E. coli DNA fragment carrying PBP6 (track 2, fig. 2b) and we conclude that this protein is a fusion product synthesised from DNA which spans the junction of vector and insert DNA at one of the EcoRI sites. It should be noted that although a protein coded by the isolated 5.85 Kb fragment has the same mobility as β-lactamase it is in fact a quite different polypeptide, since it was not precipitated with antiserum to β-lactamase (data not shown). Finally, although in some experiments using linear fragments as templates, we have observed up to 60% reductions in overall incorporation and even differential effects on specific products when compared to the equivalent supercoiled template, we conclude that this is primarily due to exonucleolytic degradation of the linear fragments rather than due to the absence of supercoiling.

Use of the in vitro system to identify specific gene products

In Fig. 3 we compare the products obtained in vitro (from the Zubay system) and in vivo (using minicells) from plasmids pBR325 and pLG282. The latter plasmid is composed of a 2.2 Kb EcoRI fragment from the ColIb plasmid carrying a gene, api, which causes abortive infection of phage BF23 in ColIb bearing bacteria (6) cloned into the single EcoRI site of pBR325. The major products from pBR325, both in vivo and in vitro, are chloramphenicol transacetylase (CAT) and β-lactamase (Fig. 3a track 2 and 3b track 1). However, in pLG282 the CAT gene product disappears as expected since this gene includes the EcoRI site, and a new polypeptide (34K) is synthesised (Fig. 3a tracks 3 and 5). A polypeptide of identical size is made in minicells containing pLG282 (Fig. 3b track 2). Functional inactivation of api in vivo by insertion of Tn5 into pLG282 resulted in the loss of this protein in vitro (track 4). Restriction of plasmid pLG282 with EcoRI and subsequent incubation of the restriction digest in the in vitro

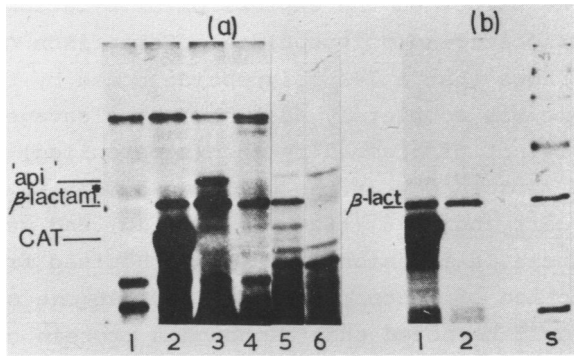

Figure 3. Identification of specific gene products in vitro. (a) track 1, no DNA; 2, vector pBR325; 3, pLG282 a derivative of pBR325 coding api$^+$; 4, pLG282: Tn5 inactivating api; 5, pLG282 cut with EcoRI; 6, purified 2.2kb fragment from pLG282 carrying api$^+$. In (b) minicells from DS410 (pBT325) track 1, or from DS410 (pLG282), track 2, were labelled with ^{35}S -methionine as in Methods; track 5, standard proteins. Note that the api protein is poorly expressed in mini-cells. The β-lactamase made in minicells appears in processed form about 2Kd smaller than β-lactamase*.

system showed that the 34K polypeptide was still synthesised (track 5) and was therefore not a fusion protein. Finally, purification of the EcoRI fragment and use of this alone to programme the in vitro system confirmed that the 34K protein was coded by this DNA and that it had been cloned together with its own promoter (Fig. 3a track 6).

As the polypeptide products described above are relatively small we also examined another plasmid, pLG215, carrying the sog gene from ColI which codes for two polypeptides of 240K and 180K, the larger peptide has been shown to have DNA primase activity (7). When the products of this incubation were analysed, two proteins of approximately 240K and 180K were present and these comigrated with the products obtained from minicells carrying pLG215 (results not shown). It is reassuring to know that large peptides may be identified just as easily as small ones using this system.

Use of DNA fragments *in vitro* to identify polypeptides coded by cloned sequences which are similar in molecular weight to polypeptides coded by the vector

A small 2.5kb EcoRI fragment from the 2 min region of the *E. coli* chromosome, carrying the gene *envA* was cloned into the vector pKN410 derived from RI (19). When the products obtained after incubation of this plasmid (pLG510) in the *in vitro* system (Fig. 4 track 2) were compared with those programmed by the vector pKN410 above (track 3) no clear difference was observed. Plasmid DNA pKN410, directed the synthesis of two polypeptides with mol wt 31.5K and 32.5K, and the 31.5K peptide was shown to be β-lactamase by immunoprecipitation. Restriction of pKN410 with EcoRI and subsequent incubation *in vitro* resulted in the disappearance of the 32.5K polypeptide, but similar restriction of pLG510 had little effect on the products (results not shown). Finally, purification of the 2.5Kb EcoRI fragment and incubation of this DNA fragment *in vitro* showed that a 32K polypeptide was coded by this region, complete with its own promoter (Fig. 4 track 1). A recent study (20) indicates that this protein is probably the

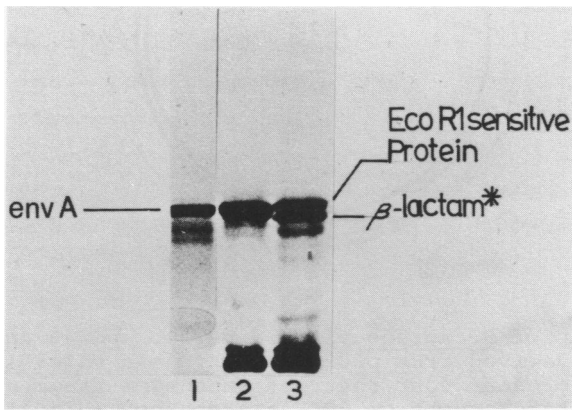

Figure 4. Identification of the *E. coli* *envA* product. Polypeptides were labelled in the *in vitro* system using the S30 extract from MRE 600 and analysed as in Fig. 1. Track 1, using a linear 2.5 Kb EcoRI fragment from pLG510; 2, pLG510 derived from the vector pKN410; 3, pKN410.

envA product.

Mapping polypeptides on a Staphylococcal plasmid template

The last four sections have highlighted the versatility of the E. coli transcription/translation system in the identification of gene products. By investigating the ability of the E. coli S30 extract to be programmed by heterologous DNAs we hoped to extend its usefulness. pC221 is a naturally occurring plasmid of Staphylococcus aureus and codes for a chloramphenicol transacetylase (CAT). Two smaller, well characterised derivatives of this plasmid, pCW46 and pCW41 (which also code for CAT), were also available (21). The relationship of these two derivatives to pC221 is shown in figure 5. DNA of these

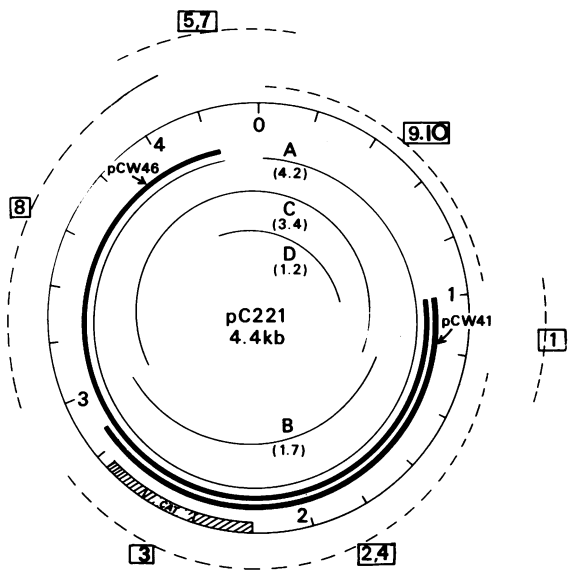

Figure 5. Map of S. aureus plasmid pC221. pCW46 and pCW41, two plasmids derived from pC221 are shown in thick lines. Thin lines represent four restriction enzyme fragments (sizes in Kb in brackets) cut from pC221; A an EcoRI-HindIII fragment; B, HpaII fragment, C, HpaII fragment; D, MboI fragment. As a result of the in vitro analysis of proteins programmed by all these DNA templates shown in Fig. 6, the approximate location (broken lines) of 9 polypeptides is shown in the boxes. Polypeptide 3, which is coded by all three plasmids and fragment B was deduced to be the CAT gene product.

three plasmids and several purified restriction fragments of pC221, A, B, C and D was prepared and incubated with the E. coli in vitro system. Incorporation of radioactivity into TCA precipitable counts showed that the S. aureus DNA appeared to be just as efficient as E. coli DNA in programming the system. The results obtained after analysis of the peptide products by SDS-polyacrylamide gel electrophoresis and subsequent autoradiography, are shown in figure 6. The different restriction fragments gave rise to a unique set of polypeptides and combined with the results from the whole plasmids we were able to assign at least 9 polypeptide products to specific regions of the parent plasmid (figure 5). The 26.5 Kd polypeptide, protein 3 on fragment B, has been tentatively identified as the CAT protein, since DNA sequencing studies of the CAT gene indicate a calculated molecular weight of 25.9 Kd (W.V. Shaw, personal communication).

DISCUSSION

Despite some earlier indications (17) that supercoiled

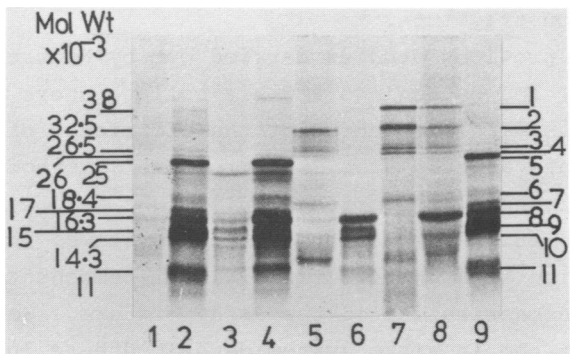

Figure 6. Polypeptides programmed by S. aureus DNA in vitro. Equimolar amounts of S. aureus supercoiled plasmid DNA or linear fragments were used to programme the in vitro system using an S30 extract from MRE600, Track 1, no DNA; 2, pC221; 3, MboI fragment D; 4, HpaII fragment C; 5, HpaII fragment B; 6, EcoRI-HindIII fragment A; 7, pCW41; 8, pCW46; 9, pC221. Two polypeptides, 6 and 11 could not be unambiguously assigned to a position on the pC221 map shown in Fig. 5. Polypeptides 1 and 2 appeared to be expressed more efficiently from the small plasmids than from pC221 or restriction fragment A.

DNA might be essential for efficient expression of many genes, our results show that under the *in vitro* conditions used here, supercoiling is not an important factor. Since local melting of the double helix, a critical step in the initiation of transcription, is thought to be dependent upon temperature and ionic strength in addition to supercoiling, the relatively low ionic strength, about 50mM in the reaction mixture used here, may render supercoiling relatively unimportant. Freed from the constraint of an essential requirement for supercoiling the *in vitro* system becomes an immensely powerful tool for the unambiguous localisation of gene products and their corresponding promoters to quite small regions of DNA, e.g. a given restriction fragment of a phage or a plasmid. In addition, elimination of a specific polypeptide product by prior treatment of the template with a restriction enzyme can be used to identify intra-gene restriction sites; advantage could be taken of this to match polypeptides eliminated in this way with previously unidentified genes by subsequent genetic analysis of sub-clones carrying these new restriction fragments.

Although previous studies carried out by Nomura and co-workers (4) and by Konings and his collaborators (3) have provided similar evidence for the efficiency of linear fragments as templates *in vitro*, their methods have not been generally adopted. This might be due in part to the relative complexity of those systems, involving the purification of a number of individual components and their final reconstitution in the assay mixture. We wish to stress that the *in vitro* system described here (provided the method of preparation is followed carefully) is extraordinarily simple and convenient to use. In addition, whilst direct comparisons are difficult to make because fewer details were published in the earlier reports, the extremely short autoradiographic exposure times required, as little as a few hours in some cases, testifies to the efficiency of the present system.

S-30 extracts have now been prepared successfully from

several different E. coli (all RNase$^+$) strains in this laboratory and the extracts are quite stable for at least a year at -80°C. By preparing large initial stocks of both the S-30 extract and the low molecular weight mix we ensure perfect reproducibility of the assay and the identification of the polypeptides coded by a few µg of a given DNA sequence then becomes a simple matter of mixing the various components, incubating in the presence of [^{35}S]-methionine and analysing the products by standard SDS-PAGE procedures. We hope to extend the use of the E. coli system to a wide range of bacterial DNAs and the results obtained with S. aureus DNA encourage us that this might be quite successful. Preliminary results indicate that B. subtilis DNA is also faithfully and efficiently translated in the E. coli system (Sarah Eccles, personal communication). The ability to produce Zubay extracts from an unlimited range of E. coli K12 mutants should also greatly facilitate studies of the control of gene expression. This possibility, together with attempts to produce an efficient in vitro system for other gram negative organisms are now being explored.

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