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Solubilization and immune-detection of β-galactosidase hybrid proteins carrying foreign antigenic determinants

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ABSTRACT

Three DNA fragments representing almost the entire *E1* gene of Semliki Forest virus (SFV) were inserted into a *cro-lacZ* expression vector by oligo dC.oligo dG tailing. Fragments inserted close to the 5'end of the *lacZ* gene gave rise to hybrid proteins which were rapidly degraded. Insertion of the same fragments at the 3'end, however, resulted in the synthesis of stable hybrid proteins which precipitated in an insoluble form within the bacteria. Insufficient hybrid protein was soluble to allow detection by immunoradiometric assay. Colonies grown on nitrocellulose filters, however, could be solubilized in SDS and subsequently renatured such that antibodies raised against the intact or SDS-denatured E1 protein cross-reacted with the hybrid proteins in a high percentage of colonies. This model system demonstrates a simple procedure for identifying DNA exon fragments by the immunological detection of expressed hybrid proteins.

INTRODUCTION

A problem which is frequently encountered in attempts to clone cDNA from long or low abundance mRNA molecules is that *in vitro* translation of mRNA, purified by hybridisation to cDNA clones, cannot provide a strong enough signal for identification of positive clones. A possible way round this problem is to express the cDNA in *E.coli* and detect the foreign antigenic determinants *in situ* using appropriate antibodies. In this way screening of clones is performed in a single step without necessitating *in vitro* translation. The construction of expression vectors has been described (1) which contain all the neccessary signals for efficient transcription and translation in *E.coli* and which can direct the synthesis of up to 30% of the SDS-extractable protein as a β-galactosidase fusion protein . One property of these hybrid β-galactosidase proteins synthesised at high levels is

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that they precipitate within the bacterial cytoplasm. While this decreases the likelihood of proteolytic degradation (2) it also makes the detection of expressed antigenic determinants difficult since most of the protein is not in solution. In this paper a simple method is reported for the solubilization of these hybrid proteins such that they will react with appropriate antibodies.

MATERIALS AND METHODS

Bacterial strains and plasmids

Transformations were performed using strain K-12 ΔH1Δtrp which is a lacZ⁻am strain carrying the defective λ prophage λNam7Nam53cI857ΔH1 (3). Recombinants were grown at 30°C for plasmid preparations, under which conditions the P_R promoter is repressed, and were scored for the presence of an E1 fragment by restriction enzyme mapping of the isolated plasmids using the endonuclease Hinf I. Hybrid protein expression was induced by incubating plates or cultures for 90 min at 42°C.

pCL547 was constructed by deleting the Eco RI site upstream from the P_R promoter of pCL47 (1) as shown in figure 1. A fragment containing the P_R promoter and cro segment was cut from pCL47 by digestion with Eco RI, filling in the protruding ends with the Klenow fragment of E.coli DNA polymerase I, and then digestion with Bam H1. This piece was ligated into pBR322 which had been treated in a similar manner. In a second step the Pst I.Bam H1 fragment from this recombinant containing part of the β-lactamase gene, the deleted Eco RI site, the P_R promoter and the cro segment was recombined with Pst 1.Bam H1 fragments from pCL19ΔY-T. This vector is similar to pCL47 except that it has 8 additional base pairs of cro and tandom transcription terminators from phage fd inserted after the lacZ gene. pCL547 was selected from the recombinants on the basis of ampicillin resistance, lac⁺ phenotype, size of the plasmid, and digestion products using Eco RI. All methods used in the construction were as previously described (1).

Detection of hybrid proteins

SDS-polyacrylamide gel electrophoresis was performed as previously described (4,1).

Antiserum against the E1 glycoprotein of Semliki Forest virus was

raised in rabbits using the E1 polypeptide cut out and electroeluted from SDS gels as previously described (5). Antiserum was also raised against the 4.5S complex of spike proteins and affinity purified on a column of Con-A Ultrogel to which the 29S complex of spike proteins had been covalently bound (5).

Immune electron microscopy of thin frozen sections was performed by Gareth Griffiths using the method of Tokuyasu (6) as previously described (7).

'Colony' blots were performed as follows: Colonies grown on nitrocellulose fiters (S&S BA85/20) were solubilized by laying the filters onto 3 layers of 3MM paper soaked in 5% SDS and baking at 100°C for 15min. After this treatment the colonies were translucent. Electroelution of residual SDS was performed as described (8), only using the nitrocellulose filter on which the colonies had been solubilized in place of the gel-nitrocellulose sandwich. The filters were oriented with the colonies towards the negative electrode and electrophoresis carried out for 60min at 50V. The filters were then incubated in 10% horse serum, 0.2% Triton X-100 in PBS (wash buffer) as follows:

30min in wash buffer + 0.1mg DNAse/ml
3x15min in wash buffer
60min in wash buffer + specific antiserum (1-10 μg IgG/ml)
3x15min in wash buffer
60min in wash buffer + 1:2000 goat anti-(rabbit IgG) coupled to horse-radish peroxidase (Tago, Burlinggame, CA. 94010 U.S.A.)
3x15min in wash buffer
5min in PBS

The final stain was developed in 100 mM Tris.HCl pH7.5 containing 0.5mg/ml diaminobenzidine and 1:5000 hydrogen peroxide (30vols).

RESULTS

Expression of E1-β-galactosidase hybrid proteins

The construction of cro-lacZ gene fusion plasmids for improved expression of cloned genes has been described previously (1). These vectors (e.g. pCL47, figure 1) have fused cro and lacZ gene segments which may be expressed under the control of the P_R

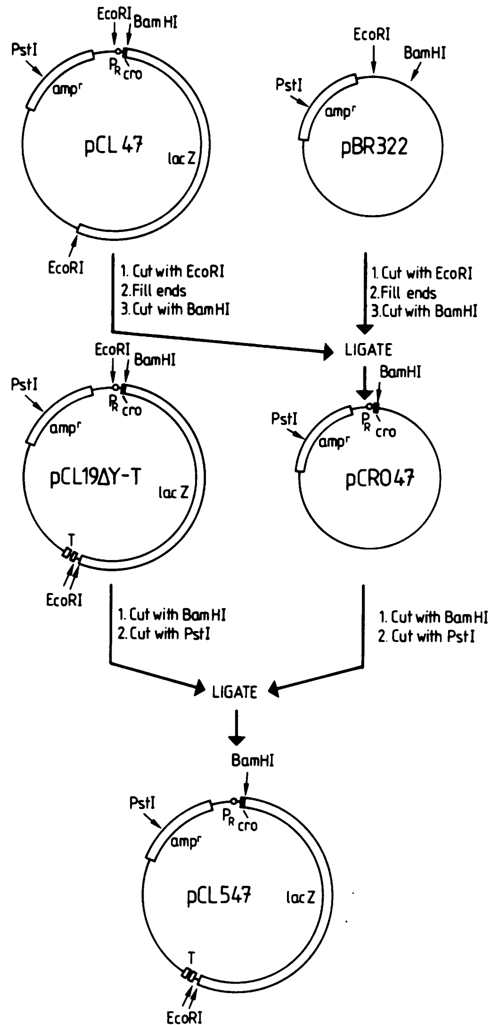

Figure 1. Schematic representation of the construction of pCL547. Arrows indicate the approximate position of restriction enxyme cleavage sites. Open boxes show the relative positions of the genes coding for β-lactamase (amp) and β-galactosidase (lacZ). T marks the position of two copies of the transcription terminator from phage fd.

promoter from bacteriophage λ. Foreign DNA may be expressed by inserting it at the unique Bam HI site in between the cro and lacZ segments. By using homopolymer dG.dC tails for inserting

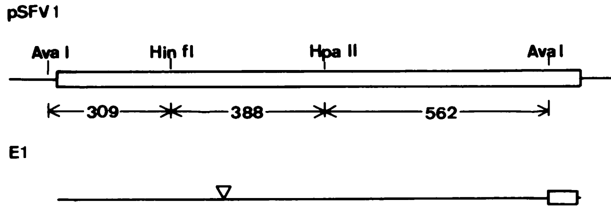

Figure 2. Fragments of the El gene. pSFV1 containing cDNA coding for the El gene of Semliki Forest virus was cut with Ava I and the 1259 base pair fragment isolated from a 6% acrylamide gel. This fragment was further cut into three pieces using the enzymes Hinf I, and Hpa II. The position of the membrane spanning domain (open box) and glycosylation site (△) on the El protein is shown.

the DNA the relative translational reading frames of insert and vector are randomised. Thus 1 in 18 recombinants should have the translational reading frame and orientation of the inserted DNA the same as both the cro and the lacZ. When E.coli harboring such recombinants is grown under conditions where the P_R promoter is active, a tripartite hybrid protein should be synthesised having a short aminoterminus (6 amino acids for pCL47) derived from cro and a carboxyterminal domain of β-galactosidase. This domain is enzymatically active and was shown in previous studies with cro-lacZ fusions to be unaffected by the length of the cro polypeptide (1). It was hoped therefore that in-frame fusions could be screened on the basis of their lac^+ phenotype. For this purpose a cro-lacZ vector in which the translational reading frames of cro and lacZ were out of phase (pCL19△Y-T, figure 1) was used so that recombinants could be picked up against a lac^- background.

As a model system for screening cDNA fragments by immunological detection of hybrid proteins expressed in vivo, the El gene from Semliki Forest virus was chosen as a source of DNA, since a plasmid containing the gene and several antisera were available (5,9). Three fragments of the El gene (figure 2) were made by Hinf I and Hpa II digestion of a 1259 base pair Ava I fragment of pSFV1 (9), which contains all but the terminal 75 base pairs of the El gene (coding for the membrane spanning region of the protein) and an additional 20 base pairs at the

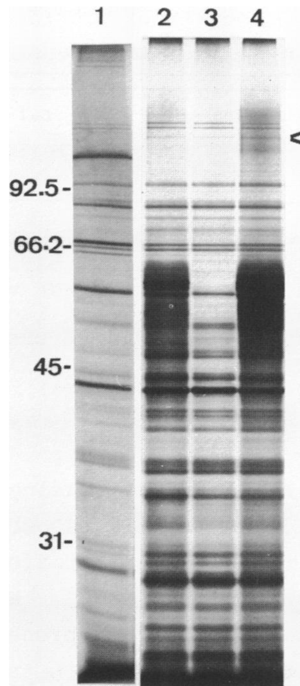

Figure 3. Products of cro-E1-lacZ fusions. Recombinants obtained by inserting E1 fragments into the Bam H1 site of pCL19ΔY-T were grown at 30°C in 2ml cultures overnight, diluted 1:200 in L.broth containing 100μg ampicillin/ml, and then induced for 90min at 42°C when at a concentration of 5×10^7cells/ml. 20 μl of culture was then electrophoresed on a 10% polyacrylamide gel and silver-stained using the method of Ansorge (22). Track 1: pCL47, tracks 2-4: three recombinant cultures. The arrow marks the expected position of the cro-E1-β-galactosidase hybrid proteins. The molecular weights of marker proteins are given in kdaltons.

5'end (which code for part of the intervening 6k peptide). The majority of the E1 gene was therefore represented in the three fragments of 309, 388 and 562 base pairs. In addition it was known from the sequence of the E1 gene that only one translational reading frame was open in either orientation of the DNA for all 3 fragments. Thus only 1 in 18 full length products should be expressed when the DNA is inserted in the Bam HI site.

Figure 3 shows an SDS gel of three recombinant cultures obtained by inserting a mixture of oligo-dC-tailed E1 fragments into the oligo-dG tailed Bam HI site of pCL19ΔY-T. For

comparison the cro-β-galactosidase hybrid protein expressed by the in-frame cro-lacZ vector, pCL47 is shown in track 1. Tracks 2 and 4 are typical of 6 recombinants found in 50 studied that expressed large amounts of hybrid protein under the control of the P_R promoter (i.e. only at 42°C in a strain carrying the temperature-sensitive λ repressor, CI857). Several polypeptides are visible on the gel which have molecular weights around 60,000 daltons. No recombinants expressed a protein of the expected molecular weight (Mr=129-139,000) for an in-frame cro-E1-lacZ gene fusion and no β-galactosidase enzyme activity or antigenic determinants could be detected. A likely explanation of this result is that the 60,000 dalton polypeptides arise by proteolytic degradation of a full length hybrid protein. In support of this hypothesis is the frequency at which this type of recombinant was observed, the extreme instability of the 60,000 dalton polypeptide (which could only be observed in fresh cultures) and the trace of high molecular weight material seen on some gel tracks (arrow, figure 3).

The greatly increased instability of these cro-E1-β-galactosidase hybrid proteins compared with the hybrids expressed by the parent vectors must be caused by some perturbation of the β-galactosidase structure such that it becomes a substrate for endogenous proteases. A second series of experiments was therefore performed in which the E1 fragments were inserted into the Eco RI site close to the 3' end of the lacZ segment. In this manner the bulk of the β-galactosidase polypeptide is translated before expression of the insert DNA, hopefully establishing a normal pattern of polypeptide folding. The disadvantage of inserting DNA fragments at the 3' end of lacZ is that enzymatic activity is lost, however the increased frequency of recombinants with the cro, lacZ and E1 in the same translational reading frame and orientation (1 in 6) compensates for the inability to screen for in-frame recombinants.

In order to be able to utilise the Eco RI site at the 3'end of the lacZ the site adjacent to the P_R promoter was first deleted as shown schematically in figure 1 and described in METHODS. pCL547 is identical to pCL19ΔY-T except that it has only two Eco RI sites and 8 fewer base pairs of cro. pCL547,

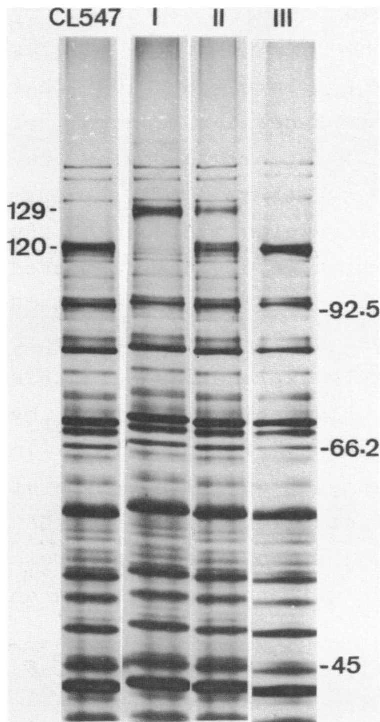

Figure 4. Products of cro-lacZ-El fusions. Recombinants obtained by replacing the Eco RI segment of pCL547 with El fragments were expressed and run on a 7.5% gel as described for figure 2. Track 1: pCL547, tracks 2-4 three recombinants containing the smallest El fragment (308 base pairs). The molecular weights of marker proteins are given in kdaltons.

like its parent plasmid, pCL47 has the translational reading frames of cro and lacZ in register and so expresses β-galactosidase activity. When pCL547 is cut with Eco RI a small segment coding for the carboxyterminal 17 amino acids of β-galactosiase is lost. This results in the loss of enzyme activity so that the background of uncut vector may be distinguished from recombinants by its lac^+ phenotype.

Figure 4 shows an SDS-gel of three types of recombinant obtained by inserting a mixture of oligo-dC-tailed El fragments into pCL547 cut with Eco RI and tailed with oligo-dG. Each of the recombinants in figure 4 contains the smallest El fragment, however three different patterns of bands were observed. Type I fusions expressed hybrid proteins with molecular weights equal to the predicted molecular weights of the cro-β-galactosidase-El hybrids (Mr=129-139,000), and occured with a frequency of 1 in 5, which is close to the frequency expected for fusions having the translational reading frames of lacZ and El in phase. Type II

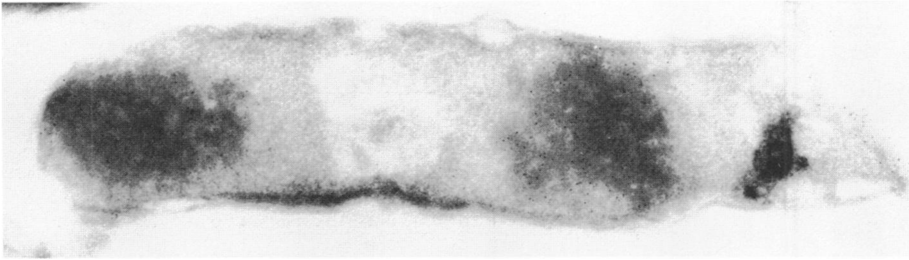

Figure 5. Immunoelectron microscopy of thin frozen sections of cro-lacZ-E1 recombinants. This photograph shows a thin frozen section of a recombinant expressing a cro-β-galactosidase hybrid protein. The cell was labelled with affinity purified rabbit anti- (SFV spike proteins) at 200μg/ml and then gold conjugated to protein A. The gold label falls over the dense precipitated material.

fusions showed two bands on an SDS-polyacrylamide gel, one with the molecular weight of the type I fusions, and one close to the molecular weight of the original cro-β-galactosidase hybrid protein. These fusions may represent fusions in which the lacZ and E1 DNA is in the correct orientation, but in which the translational reading frames are out of phase. Type III fusions had only the lower molecular weight band on SDS gels (Mr=120,000) and probably correspond to fusions with the E1 fragments in the wrong orientaion. These cro-β-galactosidase-E1 hybrid proteins were quite stable, showing no change in the size or distribution of proteins between bands when harvested cells were broken and incubated for up to 2h at 37°C before solubilization in SDS for loading on a polyacrylamide gel.

Overall 8-10% of the SDS extractable protein of the E.coli was found in the hybrid protein bands. Less than 1% of this was soluble, however, the majority being found in the pellet fraction after cells were broken using lysozyme and Triton X-100 and then centrifuged at 40,000g for 10min. In electron micrographs the bacteria were seen to contain dense bodies usually at one end of the cell, and these occasionally had areas of crystalline structure. In thin frozen sections it was possible in some recombinants to show the presence of E1 antigenic determinants in this precipitated region (figure 5). Very few gold particles were seen lying over the cytoplasm, however, and attempts to

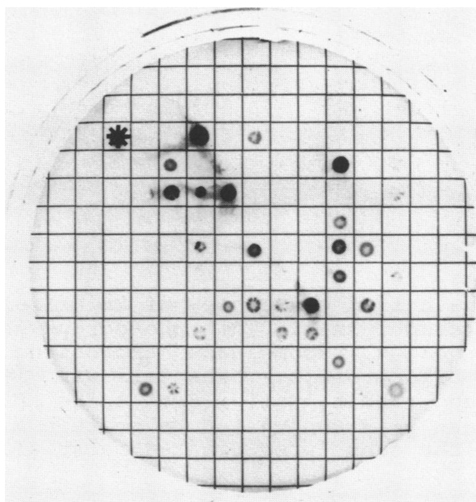

Figure 6. 'Colony' blot of recombinant cultures. 100 randomly chosen recombinants were picked in 10 rows of 10 colonies onto a nitrocellulose filter and grown at 30°C overnight. After 90min induction at 42°C the filters were removed and treated as described in METHODS. The asterisk marks the first row of 10 colonies.

detect E1 antigenic determinants in free solution were not successful despite the sensitivity of this method to as little as 10 molecules per cell (10).

Solubilization and immunological detection of hybrid proteins

Since the hybrid proteins were not found in solution after breaking open cells using lysozyme and Triton X-100, but were soluble in SDS when samples were prepared for gel electrophoresis, it seemed plausible that one route to detection of foreign antigenic determinants would be via SDS solubilization and subsequent renaturation. This was performed *in situ* on colonies grown on nitrocellulose filters as described in METHODS. The result of screening 100 randomly picked cro-lacZ-E1 recombinants is shown in figure 6. When a rabbit anti-(*E.coli* β-galactosidase) IgG fraction was used all the colonies were stained (data not shown), rabbit anti-(E1) IgG, however, stained 34% of the colonies (not all of these are visible in the photograph). 67% of these positive colonies had inserts which

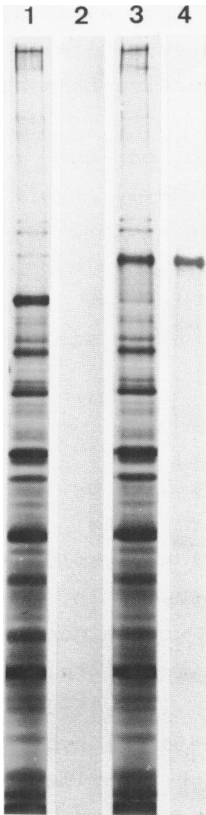

Figure 7. 'Western' blot of recombinant cultures. 5 μl samples of control and recombinant cultures after induction were electrophoresed on a 10% SDS-gel and then either silver stained or trans-blotted onto nitrocellulose. The nitrocellulose filter was then stained as described for the 'colony' blot. Silver-stained tracks: 1-pCL547, 3-type I fusion; 'western' blots: 2-pCL547, 4-type I fusion.

could be identified as one of the three E1 fragments shown in figure 2. The remainder had very small inserts and were only weakly stained. Of the recombinants having identifiable E1 inserts 50% were positive.

As the size of the E1 insert increased the expression of hybrid protein decreased. For fragment 1 with 309 base pairs the amount of hybrid protein synthesised by a type I fusion was about 75% of that expressed by the original vector. For fragment 3 with 562 base pairs this figure had dropped to 48%. In addition the amount of hybrid protein containing expressed E1 sequences was lower for type II fusions since only a fraction of the total hybrid protein expressed in this case was the full size. These levels of hybrid protein synthesis were reflected in the intensity of staining of colonies in the 'colony' blot.

Differences were also detected in the affinity of the antibody for the three E1 domains. Thus fragments 1 and 2 gave a similar intensity of staining but were considerably more reactive than an equivalent amount of fragment 3.

The immune staining of colonies was shown to be due to reaction of the antibody with cro-β-galactosidase-E1 hybrid protein by 'Western' blotting of SDS extracts of the recombinant cultures. In figure 7 it can be seen that only the hybrid protein reacts with the antibody. When type II fusions were analysed in this way only the upper, hybrid protein band stained with anti-(E1) serum.

DISCUSSION

Several attempts have been made to detect specific clones by the immunological detection of expressed antigenic determinants (10-15). These methods in general rely on the absorption of soluble antigens from lysed cells onto immobilised antibody. In few cases has the potential of enhanced expression been used since in many examples, especially of β-galactosidase fusion proteins, this leads to precipitation within the cell (1,2,16-18). Problems may also be encountered due to the degradation of hybrid proteins by endogenous bacterial proteases. In these experiments it was found that the same oligo-dC tailed DNA fragments inserted at opposite ends of the expression vector had radically different stabilities. Hybrids with DNA fragments inserted at the 5'end of the lacZ gene were extremely unstable whereas those inserted at the 3'end produced hybrids which showed no evidence of any proteolysis. This was unexpected since both short peptides and large domains expressed at the aminoterminus of β-galactosidase can form stable hybrids (1,19). It is possible that the size of fragment used in these studies was large enough to disrupt the normal folding of β-galactosidase while not being of sufficient size to form a separate domain. In addition, hybrids with E1 expressed at the carboxyterminus may precipitate more readily and so be less easily degraded by bacterial proteases (2,16-18).

The original aim of these experiments was to express a hybrid protein which could be detected in solution by immuno-radiometric assay. For this purpose a cro-lacZ expression vector

was chosen which directs the expression of sub-maximal levels of cro-β-galactosidase since more of the protein is found in this case in the aqueous phase (1). For pCL547 56% of the cro-β-galactosidase hybrid was found in lysate supernatants, and it was possible to detect β-galactosidase antigenic determinants in lysed colonies by immunoradiometric assay (10). When cells containing a cro-lacZ-E1 fusion were lysed, however, less than 1% of the hybrid protein was found in the supernatant and it was not possible to detect either β-galactosidase or E1 antigenic determinants by immunoradiometric assay. The fact that less soluble cro-β-galactosidase-E1 hybrid protein was formed despite the fact that the overall level of expression was reduced shows that precipitation is a function of the structure of the hybrid protein rather than the concentration of the hybrid protein in the cytoplasm.

Since it proved not to be possible to detect antigenic determinants in the small fraction of hybrid protein which was soluble an alternative approach was taken. This was to make use of the ability of SDS to denature and solubilize the E.coli proteins in a whole colony, and then subsequently to renature the proteins and bind them to nitrocellulose in a process similar to a 'Western' blot. Using this 'colony' blot it was possible to detect hybrids in recombinants where only low levels of hybrid protein was synthesised.

Restriction fragments of discrete sizes were used in this study so that the size and origin of each fragment could be related to its ease of detection. As the size of the E1 fragments increased the level of hybrid protein synthesis decreased. Fragments from 309 to 562 base pairs were expressed at sufficiently high levels, however, to obtain a reasonable signal to noise ratio in the 'colony' blot assay. Since this signal to noise ratio depends in part on the amount of hybrid protein synthesised, it should be possible by selecting a cro-lacZ expression vector directing the expression of higher levels of hybrid protein (e.g. pCL70 (1)) to detect larger DNA inserts. The lower limit of fragment size has not been determined, although previous studies (20) suggest that as little as 7 amino acids may be sufficient to define an antibody binding site. It

should be possible therefore to clone fragments of cDNA molecules into an expression vector like pCL547 and screen for individual clones using the 'colony' blot. When small fragments are cloned using this screening method the cloned fragment may then be used as a DNA probe for screening a library of full length cDNA.

Recombinants containing each of the three fragments of the E1 gene gave some colonies which stained with the anti- (E1) antiserum although the affinity appeared to be best with the amino-terminal domains. In these experiments essentially identical results were obtained using either the antibody raised against SDS-denatured E1 or the antibody affinity purified on intact spike proteins (data not shown), although the former gave a slightly lower background. As a general rule, however, antibodies raised against SDS-denatured antigens are preferable when detecting large proteins on 'colony' or 'Western' blots since renaturation is rarely complete. In addition the serum raised against SDS denatured antigen is more likely to bind to epitopes expressed in a hybrid protein where the folded structure of the foreign domain is unlikely to be the same as that of the whole protein. However it seems possible, since antibodies raised against both SDS-denatured and native E1 gave similar results, that antibody binding itself determines the conformation of the E1 domain of the hybrids. Another disadvantage of using an antiserum raised against a native protein is that it cannot be assumed that it will contain antibodies against epitopes all along the polypeptide chain (21). It is of interest that the central fragment was detected in these experiments since this domain would normallly by glycosylated in Eukaryotic cells. It is probable therefore that the lack of N-linked oligosaccharides in cytoplasmic bacterial hybrid proteins containing membrane or secretory protein domains will not be a major problem for screening clones by this method.

In 'Western' blots the immuno-reactive protein in recombinant cultures was identified as the cro-β-galactosidase-E1 hybrid protein. No endogenous E.coli proteins reacted with the antibody. When an antibody raised against an endogenous bacterial protein (signal peptidase) was used in the 'colony' blot all the colonies were labelled, but the level of staining

was weak compared with a type I cro-lacZ-E1 fusion stained with anti-(E1) serum. In principle therefore it should be possible to clone bacterial genes using the same screening procedure.

From the nucleic acid sequence of the E1 gene (9) it was known that only one reading frame was open in either orientation for all three of the E1 fragments. Insertions in an incorrect reading frame or orientation would be expected therefore to produce hybrid proteins which are only slightly larger than the original cro-β-galactosidase hybrid and which are unreactive with anti- (E1) sera. The frequency at which colonies containing E1 fragments were detected in the 'colony' blot was, however, 3 times higher than would be expected from random coincidence of the lacZ and E1 translational reading frames, suggesting that any fusion with the E1 fragments in the correct orientation could produce E1 antigenic determinants. This interpretation was corroborated by the analysis of protein products on SDS-gels. Both type I and type II fusions, which accounted for over half the recombinants, were capable of giving positive colonies in the 'colony' blot. Type I fusions produced a single high molecular weight hybrid protein with about the frequency expected for in-frame fusions of the lacZ and E1. Type II fusions produced two protein bands of which only the higher molecular weight band reacted with anti-(E1) sera. It is probable therefore that cro-β-galactosidase-E1 hybrid is expressed in this case by a mechanism of ribosome slippage along the homopolymer tracks at the lacZ-E1 junction.

This method of screening clones by immune detection of expressed hybrid proteins is quick and simple to perform. From the time that the colonies are picked or spread onto the nitrocellulose filters until screening is complete takes only 24h. Furthermore, the direct staining of the filter makes colony identification very easy. It should be possible to use replica filters for screening with several different antibodies or for hybridisation of the same recombinants with DNA probes.

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