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Screening for *in vivo* protein–protein interactions

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ABSTRACT **We describe an *in vivo* approach for the isolation of proteins interacting with a protein of interest. The protein of interest is "tagged" with a portion of the biotin carboxylase carrier protein (BCCP), encoded on a specially constructed plasmid, so that it becomes biotinylated *in vivo*. The "query" proteins (e.g., those in a cDNA library) are tagged by fusing them to the 3′ end of the *lacZ* gene on a λ vector in such a way that the β-galactosidase activity is not disrupted. These phage are transfected into cells containing the plasmid encoding the BCCP-tagged protein. The infection lyses the cells and exposes the protein complexes. The BCCP-tagged protein and any associated protein(s) are "captured" by using avidin, streptavidin, or anti-biotin antibody-coated filters. The detection of bound protein is accomplished by directly assaying for β-galactosidase activity on the filters. Positive plaques can be plaque-purified for DNA sequencing. We have tested this approach by using c-Fos and c-Jun as our model system. We show that avidin, streptavidin, or polyclonal anti-biotin (but not a monoclonal anti-biotin) antibody is capable of specifically capturing *in vivo* biotinylated β-galactosidase and c-Jun and that this capture is dependent upon the presence of both avidin and the BCCP moiety. Further, complexes containing c-Jun and c-Fos can also be isolated in this manner, and the isolation of this complex is dependent on the presence of c-Fos, c-Jun, avidin, and the BCCP moiety. We discuss the possible uses and limitations of this technique for isolating proteins that interact with a known protein.**

The identification of the protein(s) in a cell with which a given protein interacts is often helpful for understanding the function of that protein. A number of techniques have been devised to help elucidate these protein–protein interactions, and they fall into two main categories: *in vivo* and *in vitro* approaches.

One of the most direct *in vivo* approaches is that of immunoprecipitation. Antibodies against a target protein can be used to precipitate that protein from a cell extract along with any other proteins with which it may be complexed. Although straightforward, it often requires relatively large quantities of cell extracts in order to obtain enough of the associated proteins for further analysis (e.g., microsequencing or antibody production). Also, binding of the antibody to the target protein could displace other proteins, and the extensive washing needed to remove the nonspecific binding of more abundant proteins may eliminate rare or weakly binding proteins.

Another *in vivo* approach utilizes transcriptional activation to detect protein–protein interactions (1–4). Two portions of a DNA-binding and transcription-activating protein, which are inactive when separated, are translationally fused to a protein of interest and various cDNAs. When the two portions of the transcriptional activator are brought in "close proximity" by an interaction between the protein of interest and the protein encoded by a cDNA, a functional transcriptional activator is produced and transcription is initiated from a specific promoter. While the advantage of this approach is that it can be performed within mammalian or other eukaryotic cells (which would be expected to preserve any structure or modification of the protein needed for the interaction), one is not directly measuring the protein–protein interaction *per se* but rather the result of this interaction—i.e., transcriptional activation. It is not clear what the limits of "close proximity" are or how stable an interaction is needed for a functional transcriptional activator to be generated. Additionally, proteins which are themselves transcriptional activators might yield spurious results. Furthermore, because mammalian cells can take up multiple plasmids, even cells in which a positive interaction is observed may have only one "positive" cDNA among many "negatives." Further tests are required to identify the true positives among the more abundant negatives. Finally, experiments that depend on the clonal expansion of cells with positive interactions may select for mutations in one of the interacting proteins, especially if one of these proteins confers some growth disadvantage upon the host cell.

For these reasons, a variety of other methods to screen directly for protein–protein interactions have been devised. In general, these are *in vitro* techniques in which a protein of interest is labeled with a radioactive (5–7) or nonradioactive (8) "tag." Alternatively, an antibody against the protein of interest can serve as the "indicator" (9). This protein is then used as a probe to screen a cDNA expression library. A variety of protein–protein interactions have been identified in this way (5–14). In general, a denaturation–renaturation cycle has been employed to free any protein from complexes which might interfere with its binding to the tagged protein of interest. While helpful for some proteins, this cycle may prevent the detection of certain biologically important interactions because the renaturation steps are incapable of permitting proper refolding. In addition, a protein which requires the co-translation of its interacting protein for proper binding (15) may not be detected by this approach.

We believe that a rapid, direct *in vivo* approach to clone cDNAs encoding proteins interacting with a target protein may detect interactions not identified by the currently available *in vitro* techniques. We have developed a "double-tagging" technique which permits the detection of *in vivo* interactions between proteins. Further, because the detection system is water-soluble and can be removed quickly from the filters, repeated washing of increasing stringency can be performed to identify clones with varying degrees of affinity for the target protein. We have chosen the well-characterized Fos and Jun leucine zipper proteins as a model system to verify the feasibility of this approach.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR) Amplification of Biotin Carboxylase Carrier Protein (BCCP), c-Jun, and c-Fos and Construction of Vectors. The 3′ end of the BCCP gene, which is sufficient for *in vivo* biotinylation (16, 17), was amplified

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Abbreviations: BCCP, biotin carboxylase carrier protein; CPRG, chlorophenyl red β-D-galactopyranoside; NFDM, nonfat dry milk.

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from total bacterial genomic DNA by PCR and cloned into plasmid pMALcr-1 (New England BioLabs) between the unique *Hin*dIII and *Sal* I sites. It was also ligated in the same manner into plasmid pAX4AM+, which is identical to pAX4A+ except that the collagen sequence at the 3′ end of the β-galactosidase gene (*lacZ*) has been deleted (ref. 18; Medac, Hamburg). The presence of the BCCP gene was confirmed by restriction analysis. These recombinants were named pMALcr-1.BCCP and pAX4AM.BCCP, respectively.

The leucine zipper regions of mouse c-*jun* and c-*fos* were amplified by PCR from plasmids containing full-length murine c-*jun* and c-*fos* cDNAs (a gift from H. Vasavada, Miles Pharmaceuticals). The c-*fos* gene was cloned into plasmid pAX4AM between its unique *Eco*RI and *Hin*dIII sites. This recombinant, pAX4AM.c-fos, produced a fusion protein of the expected size. The c-*jun* gene was cloned into pMALcr-1.BCCP between the unique *Eco*RI and *Sal* I sites. This construct produced a tripartite protein consisting of MalE–c-Jun(leucine zipper)–BCCP (biotinylation domain) in *Escherichia coli* when induced with isopropyl β-D-thiogalactopyranoside.

The *lacZ*–BCCP and *lacZ*–c-*fos* hybrid genes and the unmodified *lacZ* gene were inserted into a λ phage by digesting λDASHII (Stratagene) with *Hin*dIII and ligating it with *Hin*dIII-digested pAX4AM.BCCP, pAX4AM.cfos, and pAX4AM, respectively. The ligation products were packaged and transfected into *E. coli* LE392 cells. A plaque producing β-galactosidase from each transfection was selected. The presence of the appropriate insert (BCCP or c-*fos*) was confirmed by PCR.

Preparation of Filters. Either nitrocellulose filters (Gelman) or UltraBind US450 filters (Gelman) were incubated overnight with a 1-mg/ml solution of avidin (Sigma), streptavidin (Sigma), a monoclonal anti-biotin antibody (Sigma), or a polyclonal goat anti-biotin antibody (Sigma) in 0.5 M potassium phosphate buffer, pH 7.4/200 mM NaCl/1 mM KCl. The filters were then incubated until use in 5% nonfat dry milk (NFDM) dissolved in TBST (10 mM Tris, pH 8.0/150 mM NaCl/0.05% sodium azide/0.05% Tween 20) in order to block any unreacted sites. Control filters were only blocked with the 5% NFDM in TBST. The various phage constructs were transfected into cells containing the appropriate plasmids and plated onto ampicillin plates. When the plaques became ≈1 mm in diameter (generally 3–4 hr after plating), the previously prepared filters were washed, soaked in 10 mM isopropyl β-D-thiogalactopyranoside, and then added to the plates. After 2 hr at 37°C, the filters were removed, washed three times briefly with buffer TBST, and developed with chlorophenyl red β-D-galactopyranoside (CPRG; Boehringer Mannheim) according to the manufacturer's instructions. The positive plaques generally appeared as purple-violet spots within 1 min; negative plaques took a considerably longer time to develop a less intense color.

RESULTS

Experimental Strategy. To detect a protein–protein interaction which occurs *in vivo*, one component of the interacting pair must be specifically isolated and the presence of the other protein must be detected in some way. While the isolation step could be performed in several ways, we have chosen to biotinylate the target protein *in vivo* by using a portion of the BCCP gene (17). The translational fusion of an ≈100-codon fragment of the BCCP gene to another gene generates a hybrid protein, which becomes biotinylated *in vivo* within the BCCP domain (16). This *in vivo* biotinylation of the hybrid protein enables it to be captured specifically with avidin, streptavidin, or an anti-biotin antibody.

The other component of the system requires the detection of any protein bound to the target protein. Since one will not know *a priori* what this protein is, a reporter peptide fused

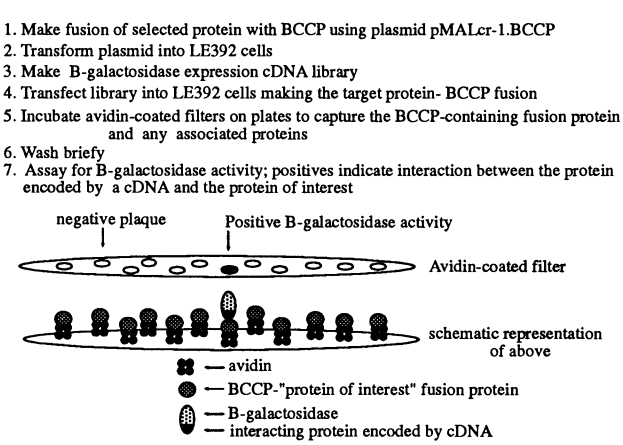

FIG. 1. "Double-tagging" assay for protein–protein interaction.

onto all the cDNAs in a library is required. We have chosen *E. coli* β-galactosidase as this reporter protein for several reasons. A number of proteins have been fused to β-galactosidase at either its N or C end; many appear to be stable and do not disrupt the activity of the β-galactosidase moiety. Many also retain their function when fused to β-galactosidase. In addition, one can assay for β-galactosidase activity directly with sensitive chromogenic substrates. However, in principle other proteins also could be used.

If the gene of any protein of interest were fused with the appropriate fragment of the BCCP gene on a plasmid, this hybrid protein would become labeled *in vivo* with biotin. Next, if another gene, or mixture of genes, were fused to the β-galactosidase gene on a λ vector, these recombinant phages could be transfected into cells carrying the plasmid encoding the hybrid protein of interest. Not only would each phage produce only one cDNA–*lacZ* hybrid so each "plaque" would represent a different cDNA, it also would lyse the bacteria and make the complexes available for capture by avidin-coated filters. These coated filters could purify the complex away from the other bacterial proteins. After suitable washing, the presence of an interacting protein could be detected by simply screening for β-galactosidase. A "positive" plaque would indicate an interaction; plaques containing proteins which do not interact with the hybrid protein of interest would appear "negative" on the filter (Fig. 1).

Extent of Biotinylation of the BCCP Hybrids. If only a small portion of the BCCP molecules were biotinylated after induction, few would be available for capture and the unbiotinylated hybrid protein would compete with the biotinylated one for its interacting protein. To test this, we performed the following experiment.

An ≈350-bp fragment of the BCCP gene was amplified from *E. coli* DNA by PCR. This was fused, in frame, to the 3′ end of the *lacZ* gene present on plasmid pAX4AM+ so that a LacZ–BCCP fusion protein was produced which preserved β-galactosidase activity (pAX4AM.BCCP). The leucine zipper portion of c-Jun was inserted into a plasmid pMALcr-1.BCCP so that a tripartite protein was produced: MalE–c-Jun–BCCP (pMALcr-1.cjun.BCCP). Both of these proteins were purified by affinity column chromatography (19, 20) and then mixed with wild-type β-galactosidase. The mixture of these three proteins then was passed over a monomeric avidin column. Most of the β-galactosidase–BCCP and MalE–c-Jun–BCCP proteins were retained on the column, whereas little or no wild-type β-galactosidase was retained (*Left*, "Elute"). Conversely, most of the wild-type β-galactosidase and little of the BCCP-containing proteins were present in the flow-through fraction (Fig. 2 *Left*, "FT"). Western blot analysis confirmed that both BCCP-containing proteins were biotinylated whereas the wild-type β-galacto-

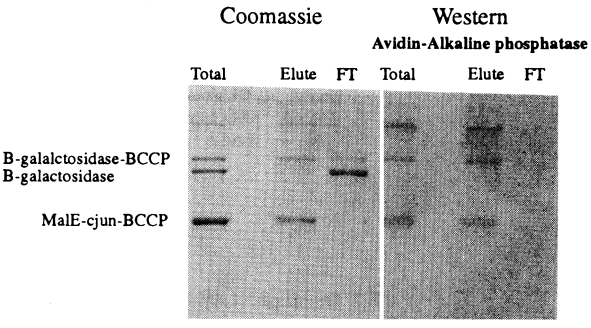

FIG. 2. Extent of biotinylation. Purified β-galactosidase, β-galactosidase–BCCP, and MalE–c-Jun–BCCP were mixed, applied to a monomeric avidin column and eluted. Aliquots from the initial mixture (Total), eluate (Elute), and flow-through fraction (FT) were analyzed by SDS/PAGE. (*Left*) Half the gel was stained with Coomassie blue. The three major protein bands in the initial mixture are indicated. The faint band near the top of the gel represents a dimer of MalE–c-Jun–BCCP. (*Right*) The other half of the gel was used for Western blot transfer of proteins to nitrocellulose. The blot was probed with avidin-alkaline phosphatase. Both β-galactosidase–BCCP and MalE–c-Jun–BCCP (and its dimer) were biotinylated (Total and Elute). The majority of the proteins not binding to the avidin column were not biotinylated. However, faint bands can be detected at the region corresponding to β-galactosidase–BCCP and MalE–c-Jun–BCCP, indicating that some fraction of these proteins which did not bind to the avidin column were nonetheless biotinylated (FT).

sidase was not. Furthermore, although densitometry of the flow-through fraction from the avidin column indicated that at least 80% of the input BCCP-containing proteins bound to the column, the Western blot analysis suggested that at least some of the protein which did not bind to the column was nonetheless biotinylated (Fig. 2 *Right*, "FT"). These data indicate that most of the BCCP-containing protein was biotinylated *in vivo*, in agreement with a previous report (16).

Binding of BCCP-Containing Protein to Avidin-Coated Filters. To test whether avidin-coated filters could specifically capture an *in vivo* biotinylated protein, pAX4AM and pAX4AM.BCCP were digested with *Hin*dIII and ligated with *Hin*dIII-digested λDASHII. The ligation products were packaged and transfected into LacZ$^-$ LE392 cells. The phage encoding the unmodified β-galactosidase was named λ.pAX4AM, and that encoding the β-galactosidase–BCCP fusion was named λ.pAX4AM.BCCP. The presence or absence of the BCCP gene in these two phages was confirmed by PCR amplification.

These phages were then transfected separately into LE392 cells. Next, an avidin-coated filter was overlaid on plates transfected with each type of phage. As controls, filters blocked only with NFDM and not containing avidin were also placed on cells transfected with each type of phage. The filters were treated as described earlier. Positive β-galactosidase activity was seen only on the avidin-coated filters placed on cells transfected with λ.pAX4AM.BCCP (Fig. 3*A*). This result showed that avidin-coated filters could specifically capture biotinylated proteins and that this capture was dependent on the presence of both avidin and BCCP: β-galactosidase–BCCP did not bind to filters lacking avidin (Fig. 3*B*), and β-galactosidase by itself did not bind to the avidin-coated filters (Fig. 3*C*).

We next mixed these λ.pAX4AM.BCCP and λ.pAX4AM phage in a ratio of 1:100 and transfected the mixture into LE392 cells. When the filter binding experiments were repeated with this mixture, only about 1% of the phage generated a positive signal, indicating that a positive signal could be detected among many negatives (data not shown).

Isolation of Protein–Protein Complexes. Having shown that it was possible to specifically capture the BCCP-tagged protein, we then attempted to show that this approach could be used to isolate protein complexes. We chose the c-Jun and c-Fos leucine zipper proteins for these studies because they have been shown to interact by one of the *in vitro* approaches already described (8).

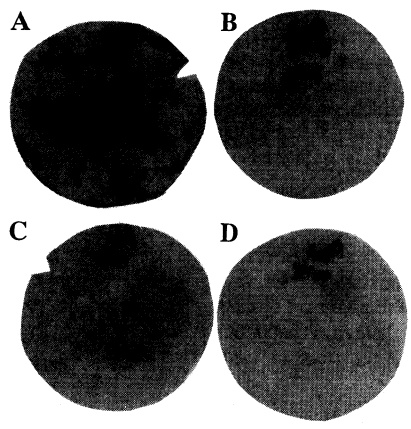

FIG. 3. Filter-binding results with *in vivo* biotinylated β-galactosidase. (*A*) Avidin-coated filter developed with CRPG after being incubated on LE392 cells transfected with λDASHII containing pAX4AM.BCCP. Intensely positive signals were observed almost immediately. (*B*) As in *A*, except that avidin was omitted. (*C*) Avidin-coated filter developed with CRPG after being incubated with LE392 cells transfected with λDASHII containing pAX4AM. Faint signals were detected only after prolonged incubation. (*D*) As in *C*, except that avidin was omitted. All filters were blocked with 5% NFDM.

The leucine zipper region of c-*jun* cDNA (corresponding to amino acids 195–334) was amplified by PCR and cloned, in frame, between the *malE* and BCCP genes on plasmid pMALcr-1.BCCP. This construct directs the synthesis of a tripartite protein: MalE–c-Jun–BCCP. After confirming that this protein was produced, we amplified by PCR the leucine zipper region of c-*fos* cDNA (corresponding to amino acids 155–279) and cloned it into the 3′ end of the *lacZ* gene on pAX4AM. This fusion protein retained both β-galactosidase activity and the ability to bind to the MalE–c-Jun–BCCP hybrid protein (data not shown).

Plasmid pAX4AM.c-fos was transferred to a λDASHII vector and filter binding experiments were performed as described earlier. The phage λ.pAX4AM.c-fos and the control phage λ.pAX4AM were transfected into LE392 cells containing pMALcr-1.c-jun.BCCP and a variety of other plasmids. When λ.pAX4AM.c-fos was transfected into cells making the MalE–c-Jun–BCCP tripartite protein, significant β-galactosidase activity could be detected on avidin-coated filters (Fig. 4*A*) but not on filters lacking avidin (Fig. 4*E*). Furthermore, avidin-coated filters placed on cells making the BCCP fusions with other proteins not known to interact with c-Jun [adenovirus E1A protein, a Tat-binding protein (TBP-1; ref. 14), or MalE alone] and transfected with λ.pAX4AM.c-fos had only background levels of β-galactosidase activity (Fig. 4 *C*, *G*, and *D*, respectively). There were approximately equal levels of β-galactosidase activity in plaques from each of these cells (data not shown). λ.pAX4AM yielded only background levels of β-galactosidase activity in LE392 cells containing pMALcr-1.cjun.BCCP (Fig. 4*B*) even though it produced ≈4 times more β-galactosidase than λ.pAX4AM.c-fos.

LE392 cells making MalE–c-Jun but lacking BCCP and transfected with λ.pAX4AM.c-fos yielded somewhat more β-galactosidase activity on avidin-coated filters than the other constructs, although significantly less than that observed when BCCP was present (Fig. 4 *F* vs. *A*). This slightly increased background may be related to the overproduction of the MalE–c-Jun hybrid in comparison with the MalE–c-

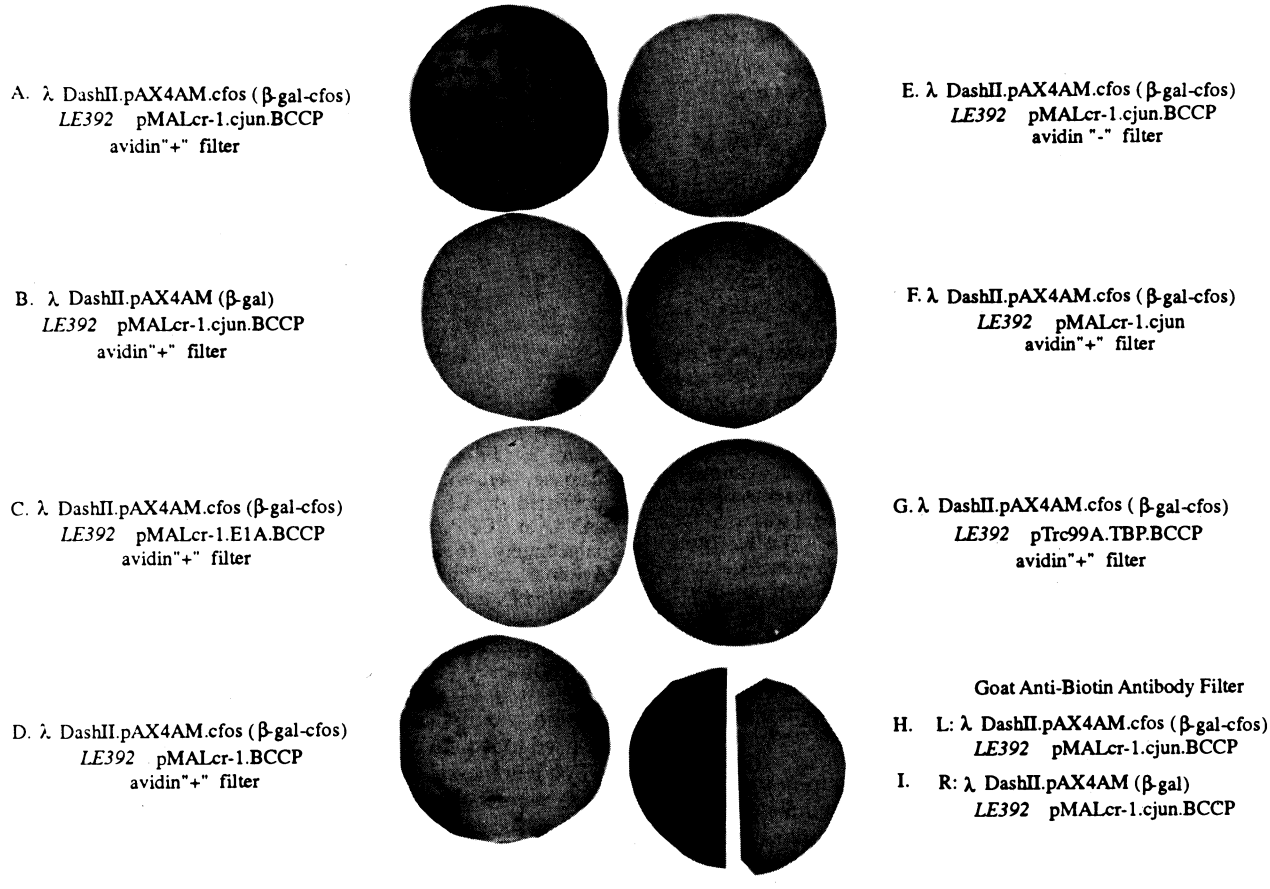

FIG. 4. Filter-binding results with c-Jun and c-Fos fusions. The top line in each section indicates the "query" protein fused with β-galactosidase on the λ vector. The second line indicates the host cell (LE392) and its resident plasmid. The third line indicates whether or not the filters were coated with avidin. For the goat anti-biotin antibody-coated filters [*H* at left (L), *I* at right (R)], no avidin was used. TBP, Tat-binding protein.

Jun–BCCP hybrid. In addition, there was also increased β-galactosidase activity in plaques when the host cell did not overproduce BCCP (an aliquot of the same phage produced significantly more β-galactosidase activity when transfected into LE392 cells containing pMALcr-1.cjun than in those containing pMALcr-1.cjun.BCCP; data not shown). The increased production of both the pMALcr-1.cjun hybrid and the β-galactosidase–c-Fos hybrid may have led to increased retention of β-galactosidase–c-Fos on the filters. When this experiment was repeated using JM103 cells (which overproduce the *lac* repressor, i.e., $LacI^q$), less β-galactosidase–c-Fos was produced and the β-galactosidase activity retained on avidin-coated filters decreased to below background.

To further confirm that these results were not caused by a spurious interaction between avidin and c-Jun or c-Fos, these experiments were repeated using filters coated with streptavidin, a monoclonal antibody against biotin, and a polyclonal antibody against biotin. Streptavidin and avidin yielded essentially identical results (data not shown). Filters coated with a polyclonal antibody against biotin gave somewhat lower background binding than did avidin (see Fig. 4 *H* and *I*); however, the monoclonal antibody-coated filters were apparently unable to capture the complex (presumably due to the inaccessibility of the appropriate portion of the biotin molecule on BCCP for this monoclonal antibody).

Mixing Study. Finally, to mimic a screening protocol, λ.pAX4AM.c-fos and λ.pAX4AM were mixed in various proportions and transfected into LE392 cells containing pMALcr-1.cjun.BCCP. The filter binding experiments with avidin-coated filters were repeated. In all instances, the frequency of positive plaques correlated with the proportion of positive and negative phage in the transfection mixture. One such example is shown in Fig. 5. One positive and negative plaque were purified to homogeneity and DNA was made from each. Restriction analysis confirmed the presence of the c-*fos* fragment in the positive plaque and its absence in the negative plaque.

DISCUSSION

We have described an approach for detecting protein–protein interactions which relies on the *in vivo* association of two proteins in *E. coli*. This technique is rapid and does not require the use of any radioactivity. Since the query proteins (i.e., those encoded by the cDNAs) have been tagged with active β-galactosidase (21), interacting proteins generate readily detectable β-galactosidase activity on avidin-coated filters when assayed by CPRG. Since CPRG is water-soluble, the assays can be repeated, after washing, using different conditions (e.g., increased salt or detergent concentration or even purified target protein). Because the other bacterial proteins are "invisible" to this assay, the presence of these other proteins does not interfere with the screening.

There are several potential limitations to this approach. Since the complexes must form within bacterial cells, certain posttranslational modifications (e.g., glycosylation or phosphorylation) or even proper folding of either protein may not occur. If these are essential for the interaction, then this assay will fail to detect an interaction. If the presence of one or more other substances (e.g., protein, nucleic acids) is required for the complex to form, or if the complex contains more than two different proteins, this approach will not be successful. In addition, it is possible that proteins which are made poorly or are not stable in *E. coli* as fusions to either β-galactosidase or BCCP may not be good candidates for this

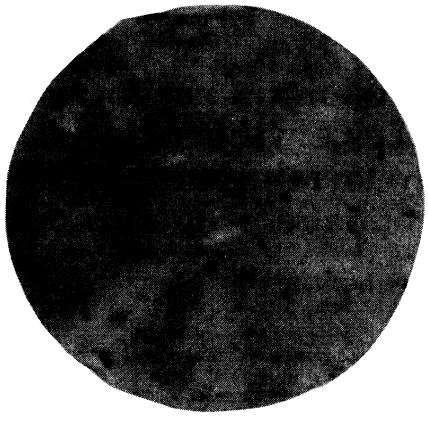

FIG. 5. Mixing experiment. LE392 cells containing pMALcr-1.cjun.BCCP were transfected with a mixture of λDASHII.pAX4AM and λDASHII.pAX4AM.cfos in a ratio of ≈200:1. After the phage plaques appeared, an avidin-coated filter was placed on the plate, incubated for 2 hr, and then washed briefly. The filter was developed in CPRG to detect the presence of β-galactosidase. The positive plaques can be identified easily.

double-tagging approach. Another possible limitation is that some proteins whose tertiary structures are significantly altered by being fused with either BCCP or β-galactosidase may be unsuitable for this assay (although it may be possible to reduce the size of both "tags" and therefore make disruptions less likely). Finally, as with all fusion protein methods, the library needs to be "deep" and one needs to screen a rather large number of plaques since, at best, only one-sixth of the plaques will produce a fusion with the reporter protein ("directional" libraries may improve this to one-third). The *in vitro* techniques already described, since they depend only upon the presence of the protein encoded by a cDNA and not necessarily as a fusion with β-galactosidase (e.g., proteolytic cleavage may destroy the fusion protein, or reinitiation may generate some protein which is not fused to β-galactosidase), may require the screening of somewhat fewer numbers of plaques.

This approach has been shown to be useful for one interacting pair of proteins and with E1A and the retinoblastoma gene product, which are also known to interact (22). Essentially identical results were obtained with regard to detection and specificity, suggesting that this approach may be useful for a wide variety of protein–protein interactions.

Although we have used CPRG for most of the assays for β-galactosidase, we also have used antibody screening with monoclonal anti-β-galactosidase antibody and have obtained similar results. Such antibody screening—which would be necessary when using λgt11 expression libraries, because the β-galactosidase activity is lost in these constructs—takes considerably longer than direct β-galactosidase screening and therefore may fail to detect protein complexes which dissociate fairly rapidly. It also has the potential disadvantage that the assay for alkaline phosphatase (which is coupled to the secondary antibody) is performed at pH 10 and may cause the dissociation of some protein complexes.

We have tried a variety of blocking agents and found that NFDM, when incubated with the filters for at least 3–4 hr, yielded the lowest background of all the blocking agents tested. In addition, we tested both avidin-coated nitrocellulose and UltraBind 450 filters. We found that the UltraBind filters yielded somewhat lower backgrounds than the nitrocellulose filters, and so they were used subsequently for all the assays. We also found that the treatment of the filters was critical. Since streptavidin and avidin seem to bind to these filters rather slowly (23), it is important to allow adequate incubation times. Short incubation periods yielded variable results. The polyclonal anti-biotin antibody yielded the highest signal/noise ratio, perhaps because IgG may bind to these filters better than avidin (23).

It may seem surprising that enough β-galactosidase activity can be recovered on avidin-coated filters to assay for it directly. However, Ruther *et al.* (24) reported that they were able to coat polyvinyl filters with an antibody (anti-lysozyme) and detect, by β-galactosidase activity, fusions of portions of the lysozyme gene with *lacZ*. This supports our finding that anti-biotin antibody-coated filters could capture enough of the complex containing β-galactosidase-c-Fos to directly measure β-galactosidase activity. It also suggests that antibody-coated filters and an epitope tag could be used instead of the BCCP–avidin system described here.

We believe that this approach may be extended to mammalian cells, as well as DNA–protein interactions. Further, it should be useful for identifying the domains of two interacting proteins which are responsible for complex formation. It may also be useful for generating compensatory mutations in the interacting domains to identify the specific amino acid contacts.

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