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A contingent replication assay for the detection of protein–protein interactions in animal cells

(simian virus 40 large tumor antigen/GAL4 protein/herpes simplex virus protein VP16/plasmid R64/Fos–Jun complex)

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ABSTRACT **We have developed a sensitive and rapid assay system termed the contingent replication assay (CRA) for selecting cDNAs with desired functional properties from a cDNA library. The system functions in animal cells and permits enrichment of the desired cDNA in small-scale and convenient experiments. The assay can be used for the enrichment of proteins that activate transcription from conditional enhancers, bind to specific DNA sequences, or interact with target proteins of interest. In this communication we report the application of this assay to study protein–protein interactions in animal cells.**

Important progress has been made in the molecular biology of higher eukaryotes by biochemically and immunologically defining groups of proteins that associate *in vivo* with a particular protein or DNA sequence. This often involves purification of a sufficient amount of the interacting species to obtain amino acid sequence or antibodies and then using this material to clone cDNAs for the new species, a procedure that may be difficult when the abundance of the proteins is low or their interaction weak. Progress is unraveling the molecular biology of animal cells would be substantially expedited by procedures that permitted rapid cloning of cDNAs for proteins that interacted with other defined proteins or stimulated transcription of target genes *in vivo*.

Attempts have been made to develop systems in which proteins can be cloned by virtue of their ability to interact with a second protein (the "target") in yeast cells (1). Recently success has been obtained in screening λgt11 phage expression library plaque lifts with labeled target protein *in vitro* (2, 3). These procedures suffer from some limitations. In some cases the protein complex may involve or be mediated by a third component missing from the new host cell. Animal cell proteins made in bacteria or yeast may not be stable and soluble or may not have the secondary modifications necessary for their interaction. Such systems obviously do not provide the opportunity to study the effects of physiologic changes on protein–protein and protein–DNA interactions. For plaque screening, the interactions must be sufficiently stable *in vitro* to withstand washing conditions.

We describe here the evaluation of an *in vivo* approach termed the contingent replication assay (CRA) that expedites the analysis of DNA–protein and protein–protein interactions in animal cells. The approach is based on simian virus 40 (SV40) large tumor antigen (T-antigen)-assisted replication of plasmids containing cDNAs with desired properties and selective recovery of plasmids in *Escherichia coli* (ref. 4; see also ref. 5). In the present report we demonstrate the potential of the CRA to study protein–protein interactions in animal cells.

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MATERIALS AND METHODS

Plasmids and Bacteria. Parental plasmids for the various vectors used in these studies have been described (4). Fig. 1 schematically represents the new vectors. Briefly, a *Bam*HI–*Eco*RI fragment containing five copies of the binding site for the GAL4 protein (upstream activating sequence) was isolated from the plasmid G5EC (6) and cloned at the *Sma* I site of pHAV1.1 (4) to construct G5ET. Mammalian expression vectors G5EC, pSG424, and the vector producing the GAL4–VP16 fusion protein were kindly provided by I. Sadowski and M. Ptashne (6, 7). The vector for cloning random-primed cDNA fragments upstream of VP16 or for cloning oligo(dT)-primed cDNA downstream of VP16 was constructed by cloning the VP16 transactivating domain (amino acid residues 413–490) in a derivative of pSH4 (8). Various constructs containing *fos* and *jun* were obtained from V. Dwarki (9). For the construction of the R6K fusion protein, as well as for the radioactive probe, the 1.2-kilobase R6K coding region was amplified by the polymerase chain reaction from pJG101 (10). Details of the construction of the various vectors described in this article are available on request from the authors.

Mammalian Cells and Transfection. CV-1 cells (monkey kidney cell line) were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10 units of penicillin per ml, and 10 μg of streptomycin sulfate per ml. Transfections were carried out by calcium phosphate coprecipitation essentially as described by Graham *et al.* (11). For DEAE-dextran transfections, cells were grown in DMEM containing 5% Nu-Serum (Collaborative Research) (12).

Analysis of Replicated Plasmids. Extrachromosomal DNA was isolated from transfected cells 48–72 hr posttransfection essentially as described by Hirt (13) or by Birnboim and Doly's alkali/SDS lysis procedure as adapted for mammalian cells (14). Digestion was carried out with an excess of *Dpn* I (New England Biolabs, 20 units/μl) at 37°C for 4–6 hr. The digestion mixture was extracted twice with phenol/chloroform and once with chloroform, and the DNA was precipitated with ethanol. The precipitates were washed once with 70% ethanol, dried, and dissolved in 10 μl of water. Bacterial colonies were obtained by transforming plasmid DNA into Electromax *E. coli* DH10B (BRL) and plating on appropriate antibiotic-containing plates.

RESULTS

Experimental Strategy. Fig. 1 describes the overall strategy for the CRA for protein–protein interactions. It is based upon

Abbreviations: CRA, contingent replication assay; SV40, simian virus 40; T antigen, large tumor antigen; CAT, chloramphenicol acetyltransferase.

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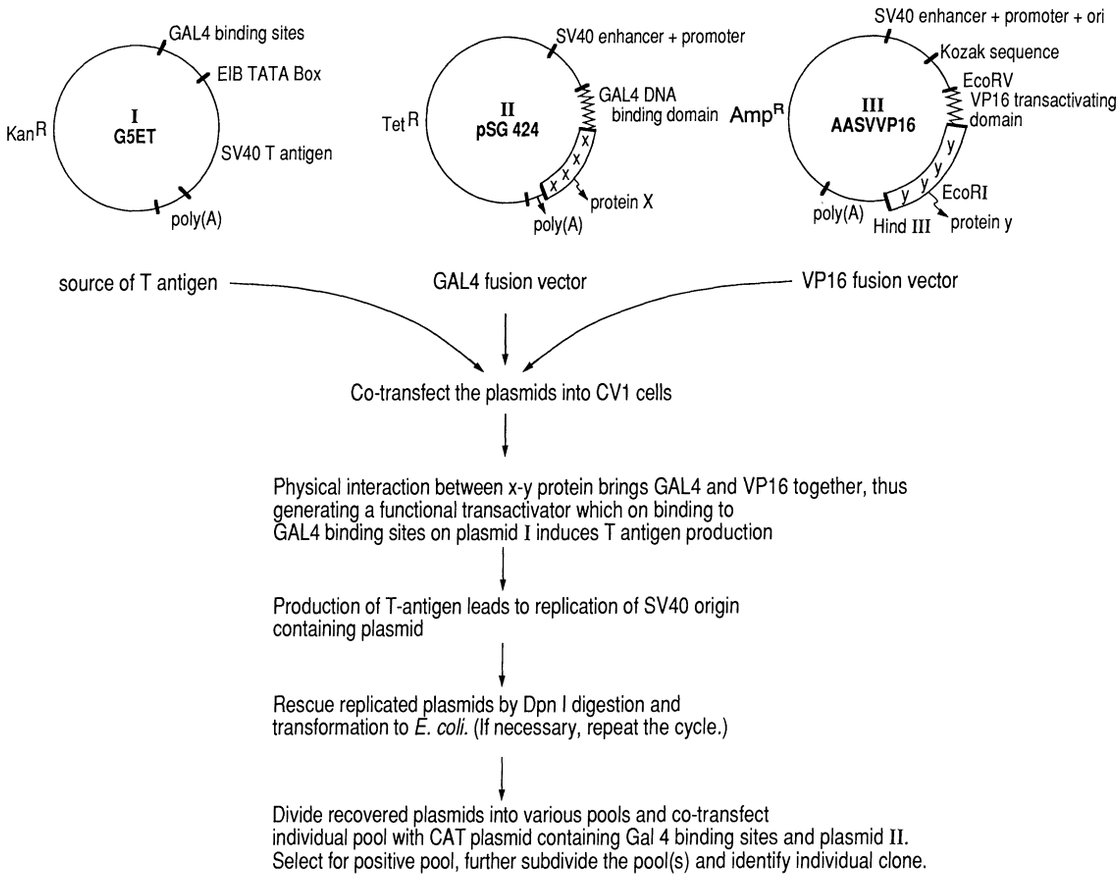

FIG. 1. Schematic representation of the CRA for protein–protein interaction. The plasmids are described in *Materials and Methods*. Kan^R, kanamycin resistance; Tet^R, tetracycline resistance; Amp^R, ampicillin resistance; ori, origin.

the following three observations. (*i*) Plasmids containing an SV40 origin of replication can replicate in permissive cells if and only if SV40 T antigen is supplied. (*ii*) Because mammalian cells do not methylate adenine residues in DNA, *Dpn* I-sensitive plasmids obtained from Dam^+ *E. coli* (which have G^mATC) become resistant to *Dpn* I when they replicate in mammalian cells. (*iii*) The DNA-binding domain of the yeast GAL4 protein, when complexed with the transactivation domain of the herpes simplex virus VP16 protein, can activate transcription from promoters containing multiple GAL4 binding sites (15–18). The activation domain of the VP16 protein is known to be a very effective activator both in animal cells and *in vitro* and to function when fused to a variety of DNA-binding peptides. The DNA-binding domain of the GAL4 protein is also known to function in animal cells (6, 18).

Three plasmids are constructed (Fig. 1). They are grown in Dam^+ *E. coli* and, in addition, treated with Dam methylase *in vitro* to ensure complete methylation. Plasmid I serves as the source of T antigen. It contains a minimal promoter that produces negligible quantities of T antigen under normal conditions. Upstream of the minimal promoter are five tandem GAL4 binding sites. When a GAL4–VP16 fusion protein is supplied, sufficient T antigen is produced to replicate plasmids containing an SV40 origin of replication (data not shown).

Plasmid II contains the sequence encoding the DNA-binding domain of the yeast GAL4 protein downstream of the SV40 promoter and enhancer. Immediately downstream of the GAL4 sequence are multiple cloning sites for the insertion of the coding sequences for any desired protein "X" (e.g. R6K), to generate a GAL4–X fusion protein. This plasmid, a derivative of pSG424, does not contain an SV40 origin of replication.

Plasmid III contains an SV40 origin of replication as well as an SV40 promoter and enhancer driving the production of the VP16 transactivating domain. Immediately downstream of the VP16 sequences are multiple cloning sites where the cDNA for any protein "Y" can be inserted to generate a VP16–Y fusion protein.

Physical interaction between proteins X and Y brings the GAL4 DNA-binding and VP16 transactivating domains together, generating a functional transactivator. This binds to the upstream GAL4 binding sequences in plasmid I and activates the transcription of SV40 T antigen. Production of T antigen in turn allows plasmid III to replicate. Replication of plasmid III causes its *Dpn* I-sensitive sites to become *Dpn* I-resistant and also increases the rate of production of the protein it encodes. After recovery of the plasmids in a Hirt lysate, restriction with *Dpn* I destroys all unreplicated plasmids. Transformation of the restricted mixture generates bacterial colonies containing the resistant, and therefore replicated, plasmids. *Dpn* I may also cut hemimethylated DNA (19), so that there will be preferential recovery of plasmids that have undergone two or more cycles of replication in the animal cells.

Evaluation of the CRA. For the present approach to work, activation of the reporter gene transcription must be dependent on GAL4–VP16 complex formation mediated by interaction between proteins X and Y. As a model system for cloning cDNAs for interacting proteins, we chose the replication protein of the plasmid R6K. This protein is known to form homodimers. It has a strong affinity for certain DNA sequences as well as a weak general affinity for DNA (10). Cotransfection of CV-1 cells with plasmid G5EC (6), which

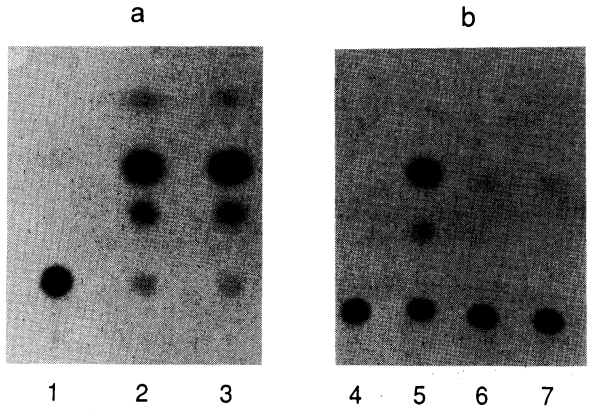

FIG. 2. CAT assay to detect protein–protein interaction. CV-1 cells (10^6 in 10-cm plates) were transfected with the indicated plasmids by calcium phosphate DNA precipitation (11). Forty-eight hours posttransfection, extracts were made by freezing and thawing five times. One hundred micrograms of protein was used for each CAT assay. Lanes: 1, G5EC; 2, G5EC plus GAL4–VP16; 3, G5EC plus VP16–GAL4; 4, G5EC; 5, G5EC plus GAL4–R6K plus VP16–R6K; 6, G5EC plus GAL4–R6K; 7, G5EC plus VP16-R6K.

contains a chloramphenicol acetyltransferase (CAT) reporter gene downstream of GAL4 binding sequences, and a second plasmid, expressing either a GAL4–R6K or a VP16–R6K fusion protein, showed that the fusion proteins did not augment production of CAT (Fig. 2, lanes 4, 6, 7). But when all three plasmids were transfected together, there was a significant increase in the production of CAT (lane 5). There was minimal activation of a CAT gene unless the gene was linked to upstream activating sequence, so that the weak nonspecific DNA affinity of the R6K protein did not interfere with the assay (data not shown).

The CRA was performed with the same set of plasmids except for the reporter gene, where the CAT gene was replaced by the SV40 T antigen. Transfection of the reporter plasmid (G5ET) with either plasmid II or plasmid III separately did not lead to increased expression of T antigen as measured by the replication assay (Table 1, Exps. 1–5), nor did transfection with all three plasmids if R6K coding sequences were removed from either plasmid II or plasmid III. Control experiments (Table 1, Exps. 1–5) demonstrated essentially similar results as those obtained by CAT assay: that is, when and only when the reporter plasmid had GAL4 binding sites and cells were cotransfected with a plasmid expressing both GAL4–R6K and VP16–R6K, there was a significant increase in the number of bacterial colonies, which was a measurement of plasmid replication in CV-1 cells. These controls also indicated that the background was sufficiently low to permit the detection of protein–protein interactions in a small fraction of the transfected cells. Further, the VP16 activation domain can activate the expression of reporter gene (linked to GAL4 binding sites) with approximately equivalent efficiency when fused to GAL4 sequences as an amino- or carboxyl-terminal extension (Fig. 2, lanes 2 and 3).

After establishing that physical interaction between R6K fusion proteins can bring the DNA-binding domain of GAL4 and the transactivating domain of VP16 together to generate a functional transactivator, we performed a series of experiments to test the ability of CRA selectively to recover an R6K–VP16 fusion cDNA from a mixture containing an excess of VP16 cDNA expression plasmids. The results of three such experiments are summarized in Table 1. When cells were transfected with a mixture of R6K–VP16 plasmids with large excesses of plasmids that expressed VP16 without R6K (i.e., AASV VP16) there was consistently an increase in the number of bacterial colonies recovered as compared with cells transfected with a mixture of plasmids but without VP16–R6K. The recovery of plasmids containing R6K sequence was evaluated by performing colony hybridization of the recovered bacterial clones with an R6K probe. The ratio of hybridization-positive colonies (column 7) to the total number of colonies obtained from recovered plasmids (column 6) and the corresponding ratio in the input mixture (columns 4 and 5) were used to calculate the enrichment. For example, in Exp. 10, the input mixture had 1 molecule of R6K plasmid per 10^4 molecules of AASV VP16 plasmid, while the recovered material had 224 R6K-positive colonies out of 8900 *Dpn* I- and ampicillin-resistant colonies; thus the enrichment was about 250-fold. In this experiment, DNA was prepared from 24 colonies that hybridized to an R6K probe. Eighteen of these clones retained a full-size R6K insert. Even after correction for this, enrichment for VP16–R6K was over 180-fold. In addition, the data indicate that the recovery of the desired plasmid (AASV VP16–R6K) actually increases as its amount in the initial transfection mixture decreases (Table 1, Exps. 7–10), raising the possibility that some type of "squelching" or other unknown interference phenomenon may be occurring (6, 18).

To confirm the general applicability of the CRA, we performed experiments with a series of Fos and Jun constructs. Fos and Jun are two sequence-specific DNA-binding

Table 1. R6K–R6K interaction

Exp.	Plasmid DNA,* μg				Total no. of *Dpn* I^R and Amp^R colonies	No. of colonies containing R6K inserts
	G5ET (Kan^R, −)	GAL4–R6K (Amp^R, −)	VP16–R6K (Amp^R, +)	AASV VP16 (Amp^R, +)		
1	5	5	–	–	603	
2	5	–	5	–	409	
3	5	–	–	5	209	
4	5	–	5	5	118	
5	–	–	–	5	320	
6	5	5	5	5	23,500	743
7	5	5	0.5	5	26,200	954
8	5	5	0.05	5	19,200	2,074
9	5	5	0.005	5	24,000	2,496
10	5	5	0.0005	5	8,900	224

CV-1 cells were plated to 60% confluency (6–8 × 10^5 cells per 10-cm plate) in DMEM containing 5% Nu-Serum. Cells were transfected with the indicated amounts of plasmid DNA by using DEAE-dextran (400 μg/ml for 60 min), essentially as described by Aruffo and Seed (12). Forty-eight hours later a Hirt lysate was prepared and replicated plasmids were scored. Plasmids used in these experiments are described in Fig. 1 and ref. 4. The radioactive probe to screen for R6K insert was generated as described in *Materials and Methods*.

*Antibiotic resistance and replication competence (+) or incompetence (−) are indicated in parentheses.

proteins that interact with one another by means of leucine zippers and activate transcription when bound to AP1 sites upstream of promoters (20). An expression plasmid containing the entire *fos* gene downstream of the GAL4 DNA-binding domain (GAL4–Fos) was cotransfected into cells in various combinations with a second plasmid that expressed a VP16–Jun fusion protein and had an intact SV40 origin of replication. All transfections included a plasmid that contained the T-antigen gene downstream of GAL4 binding sites, and simultaneously a second plasmid expressing VP16 activating sequences without any fusion protein. This last plasmid was SV40 ori$^+$ and its replication served as a separate measure of the amount of effective T antigen produced. When the GAL4–Fos fusion protein was expressed from a replication-competent plasmid, relatively large amounts of replicated plasmid were recovered (Table 2, row 1), presumably because the low level of transcription stimulated by GAL4–Fos increased as plasmids replicated and the level of GAL4–Fos templates and their expression increased. When the GAL4–Fos fusion protein was expressed from a replication-incompetent plasmid, replication was about only 2-fold above background level (Table 2, compare rows 2 and 4). In addition, transfection with a VP16–Jun expression vector in the absence of Fos did not increase replication (Table 2, row 4). Cotransfection with a VP16–Jun expression plasmid and the GAL4–Fos plasmid produced an ≈10-fold increase in the number of plasmids recovered. This demonstrated the efficacy of the CRA in a second system, involving proteins that may be expressed physiologically in the host cell.

To evaluate the applicability of the CRA to study interactions of proteins already present in significant amounts in the host cell, we prepared VP16 and GAL4 amino-terminal fusions to the cDNAs for either the RAP30 or the RAP74 subunit of the general transcription factor TFIIF (also known as FC) (21–23). Again, a prominent stimulation of transcription was observed on cotransfection of a VP16 fusion of one protein and a GAL4 fusion of the other. (T. Aso, H.A.V., F.J.G., and S.M.W., unpublished results).

DISCUSSION

We have described an approach to study protein–protein interactions in animal cells. We demonstrate that it is possible to obtain at least 100-fold enrichment of cDNAs encoding proteins involved in such interactions from the large excess of other cDNAs. Because of such an enrichment, it is possible to divide the recovered colonies into a small number of pools, to perform batch screening, and eventually to identify the desired cDNAs. In earlier experiments involving the bovine papilloma virus conditional enhancer (LCR region) and its transactivating protein E2, we have shown that it is possible to recover cDNA clones encoding desired transcriptional activators from a complex cDNA library. Thus, the CRA can be adapted to study both DNA–protein and protein–protein interactions in animal cells (refs. 4 and 5; H.A.V., S.G., and S.M.W., unpublished results). The CRA appears relatively insensitive to interference by proteins with nonspecific moderate levels of affinity for DNA, so that it could be used to screen for proteins binding to larger DNA probes than are customarily used in screening phage expression libraries. This decreases the need to perform preliminary experiments to define recognition sequences and may permit the analysis of more physiologic complexes involving extended arrangements of recognition sequences and binding proteins.

Table 2. Fos–Jun interaction

G5ET (Kan^R)	GAL4–Fos (Amp^R)	VP16–Jun (Amp^R)	AASV VP16 (Amp^R)	Total no. of *Dpn* I^R and Amp^R colonies
+	+	−	+	21,000
+	+*	−	+	660
+	+*	+	+	3,130
+	−	+	+	320

The details of transfections and analysis of replicated plasmids are described in Fig. 1 legend or in *Materials and Methods*.
*pSG424-Fos plasmid was digested with *Sfi* I and treated with T4 DNA polymerase to impair the replication of this plasmid in mammalian cells. Other details are as in Table 1 legend.

The CRA has advantages over conventional expression systems for animal cells. It is potentially very sensitive since the level of T-antigen expression and hence vector replication will in general increase as more activator protein is made. The assay is also relatively rapid, requiring only transient transfection assays. Cloning of the enriched cDNAs is done directly without imposition of any secondary selection or fractionation on the expressing cells. The assay, being a plasmid-based replication system, avoids the packaging constraints in systems dependent on transmission of virions from cell to cell. Also, because no cell-to-cell transmission of DNA occurs, rare and rapidly replicating variants cannot outgrow the desired constructs.

The present procedures are fairly well optimized for recovery of transfected DNA and other steps in the manipulation. The results of the dilution experiments indicate that a small number of tissue culture plates would be sufficient to recover a cDNA corresponding to an mRNA present at 1 part in 10^4 in the total poly(A)$^+$ RNA population, even when dilution by cDNA inserts that are out of frame with the VP16 codons or inversely oriented in the cDNA expression vector are taken into consideration.

The general limitations in the procedure are the extent of enrichment that can be obtained in a single CRA step and the number of cDNA clones that can be conveniently used in a single assay. In addition, the presence of a substantial number of background clones can make it difficult to ascertain the extent of enrichment until after the batch assays have been performed. Some of these clones may come from the carryover of nonreplicated plasmid that resisted cleavage with *Dpn* I even though it contains many G^{m}ATC sequences. Further methylation of plasmids grown in Dam$^+$ bacteria with Dam methylase ensures the complete methylation and thus susceptibility to *Dpn* I. Methylation of plasmid DNA with Dam methylase prior to transfection of tissue culture cells produced a rather remarkable reduction in background, ranging in different experiments from 100-fold to over 500-fold, and reduced background to a few hundred bacterial colonies per three tissue culture plates. This increases the purification obtained in a single cycle of CRA to the point where it should generally not be necessary to use more than one cycle or to perform batch screening to identify the desired cDNA. The low background has another very practical advantage. Even when a scarce cDNA is to be rescued, it may be possible to assess the initial success of the experiment by comparing the colony count in the test sample and an appropriate control.

A second factor limiting purification by the CRA is the phenomenon of cross-feeding. Any single cell will be transfected with several types of plasmid molecules, and if any of them lead to production of T antigen, all will replicate. We have previously provided data that cross-feeding could be limited by using protoplast transfection and titrating the number of protoplasts per cell, so that a large fraction of cells would receive plasmid from only a single protoplast (24). This procedure is feasible but somewhat tedious compared with DEAE-dextran-mediated transfection.

An additional limitation of the approach for protein–protein interaction could result from dilution of the fusion protein with protein produced from chromosomal genes. This

would be more of a problem with proteins that are abundant in the host cells. Also, the present approach requires that the fusion proteins are at least partly soluble and retain their ability to interact with one another, with DNA, and with the transcription apparatus. Although theoretically a serious concern, this has not proved to be a problem in general (5, 6, 15) or with the constructs we have tested.

The SV40-based CRA described in this communication is limited to a relatively narrow range of cell types that are sufficiently permissive for SV40 replication (e.g., CV-1 or HeLa), but an analogous CRA based on the polyoma replicon should extend the method to the large range of rodent cells and somatic cell hybrids.

Both in the present R6K experiments and in earlier experiments, involving enrichment of the bovine papilloma virus E2 protein by two cycles of contingent replication, we noted that a minority of the plasmids that were positive by colony hybridization had rearranged full-length inserts. This suggests that even during replication within a single cell a significant amount of truncation or rearrangement of inserts may occur and that this could be a limitation in the application of the assay to very long cDNAs.

There are several potential applications of the CRA. For example, it may be possible to introduce fused antibody Fv segments and use the method to screen for peptide antigens (25). The assay could also be used to test for small peptides that might augment specific protein–protein interactions or bind to specific proteins, by screening a library consisting of a mixture of random 18-mer oligonucleotides cloned in the appropriate expression vector (26). Single cytoplasmic "tail" peptides or fused dimeric "tails" could be used to screen for peptides that interact with and mediate the signaling functions of particular receptors (27). Use of subtracted or normalized cDNA libraries with the CRA would decrease the size of individual experiments and expedite their completion (28, 29). Further, the approach of generating a functional transactivator by bridging the GAL4 DNA-binding domain and the VP16 transactivating domain with the help of proteins that physically interact with each other (1, 17) can be used in the screening of biological or pharmacological reagents that can dissociate or promote such interactions or to study the interactions between proteins involved in the formation of macromolecular complexes.

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