Short Technical Reports

Improved "Activator Trap" Method for the Isolation of Transcriptional Activation Domains from Random DNA Fragments

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ABSTRACT

We have previously developed an "activator trap" method for selective isolation of activation domains from viral and cellular transcription factors. In this method, random sonicated DNA fragments were ligated next to the DNA binding domain (DBD) of the GAL4 factor in a plasmid that also contained a simian virus 40 (SV40) replication origin. A library of such random insert plasmids was transfected into a monkey cell line (CV-1-5GT), which had been stably transformed with a GAL4-inducible SV40 T-antigen gene. Chimeric GAL factors with a heterologous activation domain were harvested after selective replication in these CV-1-5GT cells. Here we report a simplification and generalization of the "activator trap" method. First, the time-consuming library construction step can be omitted by direct transfection of the sonicated DNA fragments and the linearized recipient plasmid vector into CV-1-5GT cells to obtain chimeric GAL-activation factors by in vivo ligations. Second, the dependence on CV-1-5GT cells can be bypassed by direct cotransfection of all components, including a plasmid carrying the T-antigen gene into cells other than CV-1-5GT. This latter step allows the application of the method to cultured human cells, as demonstrated with the human B-cell line BJA-B.

INTRODUCTION

Eukaryotic transcription factors are composed of different functional domains that include, usually, a DNA binding domain (DBD) as well as a domain for transcriptional activation (Reference 8; for review see Reference 15). This modular structure allows the exchange of domains between different transcription factors, which is known as "domain swapping" (2). In the past, the identification of activation domains was mostly achieved by generating sets of deletion mutants and testing them for the ability to activate transcription when fused to the yeast GAL4 DBD. Recently, our laboratory has reported a novel method for the selection of strong activation domains in mammalian cells, known as the "activator trap", which has been used to isolate clones of transcriptional activators from viral genes (7). The method has the general advantage of isolating mammalian or viral activators in a homologous system, because many but not all mammalian activation domains work in yeast. Since its publication, the activator trap system has been successfully applied to isolate additional activation domains from the genome of the human cytomegalovirus (Th. Stamminger and M. Gstaiger, unpublished results). However, in the experiments of Gstaiger and Schaffner (7), the generation of a plasmid library harboring random inserts from sonicated DNA consumed more time than the rest of the procedure. Hence, we wished to further simplify the method and considered several possibilities. We wanted to test whether the random fragments and the linearized recipient plasmid vector could be cotransfected into the monkey host cells for in vivo ligation, similar to a protocol originally used to select for enhancer DNAs, the so-called enhancer trap (19). To this end, we fragmented DNA containing a potential activation domain by sonication. The resulting fragments, together with a linearized expression plasmid containing the GAL4 DBD, were cotransfected into monkey CV-1-5GT cells where in vivo ligations can join any unrelated DNA ends (Reference 19; see also References 9 and 14). Functional fusion proteins containing the GAL4 DBD and an activation domain can initiate the expression of the T-antigen gene, which is under the control of GAL4 binding sites and stably integrated in CV-1-5GT cells. The T antigen in turn induces the high rate of replication of the expression plasmid by means of the simian virus 40 (SV40) origin (References 6 and 10; for review see Reference 5). Therefore, a plasmid carrying a nonfunctional fusion protein that cannot activate T-antigen expression should not be replicated.

Here we show that the method can be simplified and used also for human cells. It is now possible simply to transfect the linearized recipient vector with the fragmented DNA containing a potential activation domain. Functional fusion proteins of GAL4 DBD and the activation domain are then generated through in vivo ligations. Plasmids encoding such a fusion protein are selectively amplified by the SV40 replication pathway and can easily be recovered.

MATERIALS AND METHODS

Activator Trap Assay with In Vivo Ligation

Plasmid DNA (40–100 µg) containing a potential activation domain was sonicated using the Branson 250 Sonifier® system (Branson Ultrasonics, Danbury, CT, USA) until most of the fragments ranged from approximately 300–900 bp. The expression vector pSCTEV-GAL4(1-93)EV has the GAL4 DNA binding domain (amino acids 1–93) under the control of the constitutively active cytomegalovirus (CMV) promoter (Figure 1A). In addition, this expression vector also has an SV40 origin of replication and can therefore be amplified with the T antigen. For in vivo ligation, pSCTEV-GAL4(1-93)EV was linearized at a unique *Eco*RV site, which is located 18 nucleotides downstream of the GAL4 DBD. No dephosphorylation reaction was performed as is normal for in vitro ligations. One microgram of linearized pSCTEV-GAL4(1-93)EV vector (0.3 pmol) and 10 µg of sonicated fragments (average length about 600 bp, 25 pmol) were transfected into CV-1-5GT or CV-1 cells on 100-mm plates using standard protocols for DEAE-dextran and calcium phosphate transfection (Reference 1 and references therein). BJA-B cells were transfected with DEAE-dextran. At 36 h after transfection, low-molecular-weight DNA was isolated (10) and digested with *Dpn*I (40 U) for 4 h. Competent DH1 *E. coli* were transformed and plated on ampicillin-supplemented plates. Plasmid DNA of individual clones was isolated, and an aliquot was digested with *Xho*I and *Hinc*II to determine the insert length. The transactivation capacity of the selected clones was determined by the luciferase assay: HeGLu cells were transfected with 1 µg of plasmid DNA per

60-mm dish using the DEAE-dextran method. This cell line, which is derived from HeLa cells, has a stable integration of the luciferase gene under the control of five GAL4 sites (see below). After 36 h, cells were washed twice with Tris-buffered saline and the luminescence was determined using the Luciferase Assay System (Promega, Zurich, Switzerland) and a Model ML 3000 Microtiter® Plate Luminometer (Dynatech, Denkendorf, Germany). Luciferase active clones were sequenced by the Sanger dideoxynucleotide technique (11).

Activator Trap Assay with In Vitro Ligation

Sonication of plasmids containing candidates for activation was performed as described above. The ends of 1.5 µg of sonicated fragments were repaired by treatment with Klenow polymerase (40 U), T4 polymerase (36 U) and T4 kinase (60 U). These fragments (1.25 µg) (average length 600 bp, 3 pmol) were then ligated with 0.5 µg (0.15 pmol) of the *Eco*RV blunt-ended, dephosphorylated pSCTEV-GAL4(1-93)EV vector using T4 ligase (40 U). After the repair reaction and ligation, phenol-dichloromethane extraction and ethanol-precipitation were performed on the samples. All the following steps were identical to the in vivo ligation assay described above.

HeGLu Cell Line

A HeLa cell line stably transformed with a GAL4-regulated luciferase gene (designated HeGLu) was constructed using p5xGAL4-luc and pacCA vectors. The p5xGAL4-luc construct contains the luciferase gene under the control of five GAL4 binding sites upstream of the TATA box of the adenovirus E1b promoter (kindly provided by Ch. Aepinus, University of Erlangen). The pacCA vector encodes a puromycin *N*-acetyl transferase under the control of the constitutively active SV40 early promoter and therefore mediates puromycin resistance (18). The HeGLu cell line was constructed as follows: 20 µg of p5xGAL4-luc and 2 µg of pacCA were transfected by calcium phosphate transfection into a 100-mm culture dish of HeLa cells. After 41 h of culture, the cells were selected for puromycin resistance (15 µg puromycin

per 10 mL medium in a 100-mm culture plate). We obtained several puromycin-resistant colonies, of which further analysis revealed one highly sensitive luciferase-responding clone named HeGLu. Transfections of as little as 16 ng DNA containing the acidic activation domain of the herpes simplex virus VP16 fused to the GAL4 DBD into HeGLu cells resulted in detectable luminescence that was 10-fold higher than background, whereas HeLa cells, in cotransfections with the GAL4 luciferase plasmid, needed approximately 60 ng to show the same luminescence.

LipofectAMINE™ Transfection

CV-1 cells were split 8 h before transfection, such that the cells were 80% confluent at the time of transfection. The transfection procedure was performed as described by Life Technologies (Basel, Switzerland). Briefly, 4.2 µL of LipofectAMINE™ Reagent (Life Technologies) were used per µg DNA. Cells were incubated for 5 h with the transfection mixture and then supplemented with medium containing twice the usual amount of fetal calf serum and calf serum (5% each instead of 2.5% each). Thirty-six hours after transfection, cells were harvested. All the following steps were identical to the in vivo ligation assay described above.

RESULTS AND DISCUSSION

Screening for VP16 Acidic Activation Domain in Random DNA Using In Vivo Ligations

The activator trap assay, as originally devised, included the generation of a library of random insert fragments in *E. coli*, which was subsequently transfected into mammalian cells for expression selection of activation domains. To avoid the amplification in *E. coli*, we decided to test if we could instead use intracellular ligation in CV-1-5GT cells. For model experiments, we chose the strong acidic activation domain of the herpes virus transactivator VP16 (3, 4,13,16,17). This acidic activation domain was cloned into the pGEX vector (resulting in pGEX-VP16AD). We fragmented pGEX-VP16AD vectors with sonication and cotransfected these fragments with linearized pSCTEV-GAL4(1-93)EV expression vector into CV-1-5GT cells (Figure 1A). To distinguish after DNA isolation between replicated and non-replicated plasmids, the plasmid DNA from CV-1-5GT cells was treated with *Dpn*I. All non-replicated plasmids are *Dpn*I-sensitive because of their bacterially derived methylation pattern; those plasmids that replicate in mammalian cells lose this prokaryotic type of methylation and therefore are resistant to *Dpn*I digestion. We obtained a total of 200 colonies in five independent experiments, of which 98 were tested individually. Twenty-five clones had a fragment integrated, of which nineteen were luciferase-positive. Sequencing of the luciferase-positive clones confirmed that they all had the expected VP16 activation domain inserted in-frame. Some of the sequenced clones were identical, reducing the number of individual clones to seven.

In these five experiments, not all plasmids that were resistant to *Dpn*I digestion (i.e., had replicated in CV-1-5GT cells) also contained an integrated VP16 activation domain. Most likely a functional fusion protein, produced from one plasmid, activated the T-antigen expression and allowed "parasite" plasmids to replicate within the same cell as well. The ratio of clones containing an activation domain to such parasite molecules was 1:5 in several experiments. To test whether the amount of parasite molecules could be reduced by a second round of transfection, we did a reconstruction experiment. Plasmid DNA with different predetermined ratios of activating-to-non- activating plasmids were extracted from bacteria and transfected into CV-1-5GT cells. With a starting ratio of 1:16, we observed only a twofold enrichment. However, in a ratio of 1:150, we obtained a 30-fold enrichment for plasmids with an activation domain. Therefore, it appears that a second transfection round in monkey cells would increase the relative yield of activating plasmids; however, it also requires a time-consuming step of amplification in *E. coli* before the second mammalian cell transfection. Therefore, this enrichment step was not routinely used.

Adaptation of the Activator Trap Assay to Cell Lines Other Than CV-1-5GT

It is known that a given activation domain can be active or inactive depending on the cell type (12), possibly as a result of specific phosphorylation and the requirement for interaction with a cell type-specific co-activator. Therefore, it seemed desirable to expand the system to other cells, without the need to generate for each experiment a cell line stably transformed with a GAL4-dependent T-antigen gene. This could only be achieved by cotransfection of all components involved, including the T-antigen gene. We therefore tested the activator trap system in monkey CV-1 cells, which do not contain an integrated copy of the T antigen. For this, 1 µg of a plasmid with the T antigen under the control of five GAL4 sites (GAL-T-antigen plasmid) was included for each

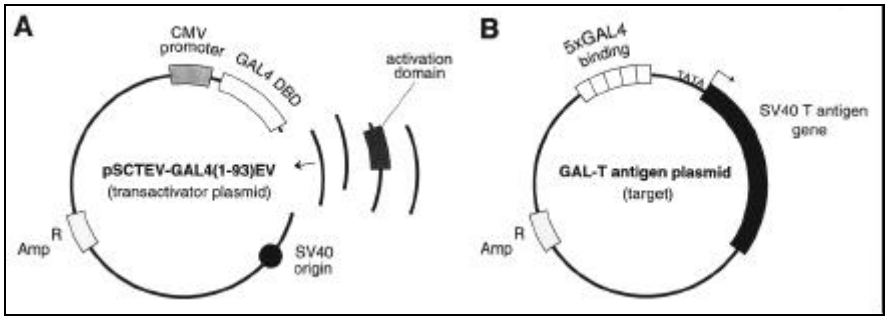

Figure 1. Map of the expression vector pSCTEV-GAL4(1-93)EV and target plasmid GAL-T antigen. A) The expression vector pSCTEV-GAL4(1-93)EV contains the GAL4 DBD (amino acids 1-93) under the control of the CMV promoter. A unique *Eco*RV site is located 18 nucleotides downstream of the DBD. In addition, this vector also has an SV40 origin of replication. Sonicated DNA fragments coding for potential activation domains are transfected with *Eco*RV linearized expression vector. B) Functional fusion proteins containing the GAL4 DBD and an activation domain can bind to the GAL4 binding sites of the target GAL-T-antigen plasmid and initiate the expression of the SV40 T antigen. The T antigen leads to the replication of the expression plasmid by means of the SV40 origin of replication. After DNA recovery, replicated transactivator plasmids were selected by digestion with *Dpn*I, which only cleaves non-replicated DNA.

cotransfection (Figure 1B). Since this GAL-T-antigen plasmid does not contain an SV40 origin of replication, it is not replicated within mammalian cells and is therefore eliminated during the *Dpn*I digestion step. In two independent cotransfections of CV-1 cells with sonicated pGEX-VP16AD vector, linearized pSCTEV-GAL4(1-93)EV expression plasmids and GAL-T-antigen plasmids, we obtained 1050 colonies in total, 22 of which were further analyzed. Seven clones contained inserts, of which two showed a relative luminescence of 0.61 and 1.14, respectively. Relative luminescence was calculated with the positive control set as 1.0. Transfection with 1 μg of the expression vector GAL4(1-93)-VP16AD or without the expression vector served as positive and negative controls, respectively. Relative luminescence of the negative control and clones without an integrated activation domain was in the same range (<0.012). The two luciferase-positive clones were sequenced and shown to represent independent insertion events with the VP16 activation domain incorporated in-frame.

To adapt the method to human cell lines, we chose human BJA-B cells (a B-lymphoblastoid cell line). This nonadherent cell line is well-transfectable using the DEAE-dextran method and, as a primate cell line, was expected to also support replication of SV40-type DNA. Using the same components as in CV-1 cells but using the DEAE-dextran transfection method, we obtained four colonies that contained an integrated DNA segment. One of these clones could activate the luciferase gene and contained the VP16 activation domain. Generally, it appeared to us that the ratio of activating-to-parasitic plasmids was less favorable in cell lines other than CV-1-5GT. In three independent cotransfections of CV-1 or BJA-B cells, we obtained a ratio of about 1:10, i.e., 10% of all analyzed clones harbored an activation domain fused to the GAL4 DB domain as compared to about 20% generally seen in CV-1-5GT transfection experiments.

In Vitro Ligation Before Transfection as a Means to Reduce the Amount of Transfected DNA

For each transfection of a 100-mm culture dish, we used between 8 and 12 μg of DNA. Normally, such DNA amounts are not critical for most cell lines but can be toxic for sensitive ones. Also, it is conceivable that not all cells have an efficient end-to-end joining mechanism to generate chimeric DNA molecules. To avoid such possible problems and to increase overall efficiency, we performed a short in vitro ligation step before transfection (see Materials and Methods). BJA-B cells were directly transfected with a ligated mixture of sonicated fragments and linearized expression vector together with a GAL-T-antigen plasmid amounting to

a total of 2.75 μg DNA. In two independent transfection experiments, we obtained two different luciferase-activating clones with a relative luminescence of 0.44 and 1.04, respectively. The same controls were used as described above. Relative luminescence of the negative control and clones without an integrated activation domain was <0.014. Both luciferase-positive clones contained an integrated VP16 fragment. This experiment indicates that it is possible to reduce the amount of transfected DNA by replacing the in vivo with an in vitro ligation. The ratio of correct clones to parasites again was about 10%, i.e., similar to the ratio observed for in vivo ligation in cell lines CV-1 and BJA-B.

Since it is known that linearized double-stranded DNA can be intracellularly trimmed or extended (14), we decided to analyze the junction of the GAL4 DNA-binding domain and the activation domain generated by in vivo ligation. In nine independent transfections, no alterations occurred at the C-terminal end of the GAL4 DBD in a total of 13 clones (of course, the VP16 junctions were all different, but their original sizes after sonication were not known). In the other four clones, deletion of one, five, seven and fourteen nucleotides were observed, respectively. This loss of nucleotides did not affect the GAL4 DBD because its 3′ end and the *Eco*RV site are separated by an 18-bp linker. Intracellular exonuclease activities are most likely responsible for this trimming. In addition, in different insert clones, we found wide variations of the spacing between the DBD and VP16 activation domain, with minimal and maximal spacers of 12 and 161 amino acids, respectively. This is in agreement with many studies in different laboratories, which have shown that chimeric GAL4 activation factors are quite forgiving towards alterations in spacing of the domains. Nevertheless, it is known that some activation domains are hidden within the natural protein and only conditionally accessible for activation. A classical deletion analysis may not reveal those constellations that alleviate such an intramolecular repression. In this respect, the activator trap assay has the advantage that a large number of random DNA segments are generated and subjected to expression selection.

Cloning of strong activation domains without prior construction of a plasmid library, just using in vivo or in vitro ligations, is now possible. Cell lines, other than CV-1-5GT can be used, as we show with human B-cells. With this method, we provide a powerful tool for the isolation and characterization of unknown transcriptional activation domains.

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Conversion of Bacterial Gene Products to Secretion-Competent Fusion Proteins

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ABSTRACT

We describe an efficient and easy procedure that allows the generation, detection and secretion of foreign proteins by the secretion apparatus of E. coli *hemolysin. The gene (or gene fragment) encoding the foreign protein (or protein domain) is inserted in-frame into a residual portion of the hemolysin gene (*hlyA*), encoding the HlyA secretion signal ($HlyA_s$). Generally, the expressed fusion is efficiently secreted into the culture supernatant of the producing strain. The new approach allows the direct generation of fusion proteins from genomic DNA fragments. The successful use of this method is demonstrated by cloning of random chromosomal DNA fragments from* Salmonella typhimurium.

INTRODUCTION

Secretion of hemolysin (HlyA) is independent of the general secretory pathway (GSP) of *E. coli* and requires a specific translocator system, which consists of two inner membrane proteins, HlyB (3) and HlyD (22), and the outer membrane protein TolC (28). The hemolysin secretion apparatus recognizes a signal sequence of 60 amino acids (aa) (9,12) or even less (13,15) that is located at the C-terminal end of HlyA. This sequence is different from the N-terminal signal sequence of secretory proteins that are transported by the *sec* pathway (21) and is not cleaved off during the secretion process. The HlyA secretion reaches maximum efficiency at logarithmic growth phase (optical density [OD_{600}] = 1–1.5). The genes containing the information for the synthesis and secretion of HlyA form an operon (7). The entire hemolysin gene (*hly*) operon, including the enhancer like regulatory element *hlyR* (27), was used to generate a secretion vector. This ColE1-derived plasmid vector carries a truncated *hlyA* cassette,