Functional Dissection of AP-1 Transcription Factors Using the Gal4 Adaptor Assay

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Abstract
In the Gal4 adaptor assay, leucine zipper-containing proteins are tethered indirectly to the promoters of Gal4-responsive reporter genes via synthetic protein chimeras consisting of a Gal4 DNA-binding domain with an attached leucine zipper. Ternary complexes composed of the DNA binding site, the adaptor protein, and a leucine zipper factor can stimulate reporter gene activity, provided that the latter component possesses a transcriptional activation domain. This system is used to assay the transcriptional function and the interactions between various AP-1 factors.

Introduction
Eukaryotic genes are expressed according to specific programs that are determined by a plethora of endogenous and environmental parameters. Thus, higher organisms face the dilemma of how to accomplish the specific regulation of tens of thousands of genes without requiring too many specific transcription factors. A solution for this problem seems to lie in the utilization of combinatorial control mechanisms with several factors acting on overlapping sets of genes. It has become clear that eukaryotic transcription control regions, often subdivided into enhancers and promoters, are ensembles of different transcription factor binding sites. Unique combinations of sequence-specific DNA-binding transcription factors can effect the regulation of a gene and specify its individual transcription profile in time and space. This concept of a modular structure of promoters and enhancers consisting of separate binding sites for transcription factors is now widely accepted (1–3).

In recent years, another variation on the theme of combinatorial gene regulation has been discovered and has since turned out to be a widespread phenomenon. Whereas the combination of binding sites determines the regulatory identity of a promoter region, in an increasing number of cases it has been found that combinations of different polypeptides (in the following referred to as "transcription factor protomers" or "protomers") are required to interact simultaneously with one of these individual binding sites. Interactions between such transcription factor protomers can occur in different ways; (a) Two different (but often related) polypeptides may have to associate through specific dimerization domains to form a dimer that gains the ability to bind to a promoter element. Well documented examples belonging to this category are the members of the Fos and Jun or of the helix-loop-helix protein families (4). (b) Two proteins can bind to adjacent DNA sequences and stabilize each other's binding activity or interact in some other way to modulate the transcription rate of the target gene. Examples of this include the interaction between the serum response factor and the ternary complex factor (5). (c) A non-DNA binding protein may have to interact with a DNA-binding factor to form a transcriptionally active complex. Such heterodimeric complexes are formed between oct-1 and the herpes simplex virus trans-activator VP16 (6).

In all cases, two (or more) different proteins are required for the transcriptional activation (or repression) mediated by a single promoter sequence element. The advantages of such "binary systems" are:
1. It appears that a given transcription factor "protomer" can interact productively with a number of functionally distinct partners. In this way, a large number of transcription factors with a variety of distinct properties can be formed by combining a few different monomers.
2. Expression of a target gene can be restricted to those situations where both components of a binary transcription factor are present. Consequently, the activity of such a gene may be more specific than that of the individual transcription factor constituents. In such a manner, a sharpening of gene activation in space, in time, or in response to extracellular inducers may be achieved.
3. Obligatory interaction of two proteins for transcription factor function generates a regulatory option that is well suited for the modulation of transcriptional responses. Negative control mechanisms are possible where a potentially active protomer is sequestered into an inactive form by interaction with an inhibitory partner molecule. Such an inhibitor would possess the dimerization domain but would be incompetent in its ability either to bind DNA or to activate transcription. Therefore, the balance between inhibitory and stimulatory partner molecules would determine overall transcriptional activity (7, 8).

To functionally understand binary transcription factor systems, it is important to determine both the transcriptional activities of the individual protomers and to assess their interaction in vivo. Here, we describe a system that is designed for such an analysis.

The example that we chose for our studies was the AP-1 transcription factor family, which is composed of Jun- and Fos-related proteins. AP-1 proteins contain a characteristic conserved amino acid sequence called the bZip domain (9, 10). This domain is bipartite; it contains a COOH-terminal leucine repeat that can adopt an α-helical conformation and serve as a dimerization interface. Interactions between two leucine repeats juxtapose
the NH2-terminal basic regions of two bZip domains to form a DNA binding-competent dimer. In vitro studies indicate that stable heterodimers can form between one transcription factor protomer of the Fos and one of the Jun class, respectively. Jun homodimers can also be formed in vitro; they are, however, much less stable than the heterodimers with Fos (11). Fos homodimers have not been found under normal experimental conditions. In vitro binding and transcription studies indicate that the predominantly active species of AP-1 are Jun-Fos-type heterodimers (12). The question of whether Jun homodimers contribute significantly to AP-1 function within the cell is still not clear. Several points, however, argue against it. Introducing Jun into undifferentiated F9 cells, which reportedly contain low concentrations of active AP-1 factors (13), gives rise to only a modest increase in AP-1 activity (14, 15), whereas strong AP-1 activity is observed only after the additional introduction of Fos into the system (16). It is not clear whether the residual activity measured after transfection with Jun alone is mediated by Jun-Jun homodimers or by heterodimers between exogenous Jun and low levels of endogenous AP-1 factors that appear to exist even in this cell system (17). Furthermore, in vitro binding and transcription studies have shown that the activity of Jun homodimers drops sharply at temperatures above 30°C, presumably as a result of “melting” of Jun-Jun homodimers (18). This indicates that they might not be stable at the temperature within mammalian or avian cells.

Due to the mutual requirement of Fos and Jun for formation of active AP-1 complexes, it is difficult to assess the transcriptional contribution of individual Fos or Jun proteins in transfection assays. Here, we use a Gal4 adaptor system to overcome this problem and to compare the transcriptional activity of different members of the Jun and Fos family independent of a heterodimerizing partner molecule.

**Results**

**The Gal4 Adaptor System.** A number of characteristic features of sequence DNA-binding transcription factors inspired the experimental approach described here. In many cases, it has been found that the DNA binding and transcriptional activation functions of transcription factors are performed by functionally independent, separable domains. As a consequence, it is possible to artificially generate functional chimeric transcription factors by combining heterologous DNA binding and transcriptional activation domains (19, 20). The activity of many DNA-binding transcription factors does not critically depend on their position relative to the start site of mRNA synthesis. Furthermore, it has been shown that a transcription factor does not even have to bind to the same DNA molecule that encodes the mRNA; it is sufficient if the factor binding sequence is brought into the proximity of the initiation site, for example, by a noncovalent biotin/streptavidin bridge (21). Similarly, transcriptional activation domains can operate when they are recruited to a target promoter indirectly, by protein-protein interactions with a different DNA-binding factor (22, 23). Thus, it would appear that the transcription initiation complex can be activated simply by the proximity of a transcription activation domain. Considering these principles, we investigated whether an AP-1 transcription factor would function when tethered indirectly to a reporter gene promoter via an artificial chimeric “adapter protein.”

The strategy behind the Gal4 adaptor system is outlined in Fig. 1. A protein chimera was designed that consists of a Gal4 DNA-binding domain and a leucine zipper derived from either c-Fos or c-Jun (Fig. 1). The DNA-binding domain of the Saccharomyces cerevisiae Gal4 transcription factor was chosen because higher eukaryotic cells appear to lack an activity with similar binding specificity. This fusion protein is devoid of any known transcriptional activation domains, so that its binding to a promoter or enhancer element would not, per se, change the transcription of linked genes (Fig. 1a).

Transcriptional activation of Gal4-responsive reporter genes may, however, be achieved by a protein possessing a transcription activation domain which can interact stably with leucine zipper of the adaptor protein (Fig. 1c). Of course, for such a system to provide reliable results, the transcription activator should fail to stimulate the reporter directly (Fig. 1b). Such an approach was indeed shown to be possible by Dang et al. (24) while our work was in progress. Eukaryotic expression vectors were constructed which encode fusions of the Gal4 DNA-binding domain and the leucine zipper dimerization motif obtained from c-Fos (Gal-FosLZ2) or c-Jun (Gal-JunLZ2). The leucine zipper cassettes utilized for these constructs possess all of the elements shown to be nec-

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1 D. Bohmann, unpublished results.

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*Fig. 1.* Strategy of the Gal4 adaptor assay. a, an adaptor protein consisting of the Gal4 DNA-binding domain (DBD) and a leucine zipper (LLLLL) binds to a GalRE but does not activate transcription. b, AP-1 like factors cannot bind to Gal4-responsive promoters and do not activate them in spite of their functional transcription activation domain (TXN). c, a complex consisting of the Gal4 adaptor and an AP-1 protein can bind to and activate the Gal4-responsive promoter.
Fig. 2. Gal4 adaptor assay on c-Fos and c-Jun in HD11 cells. Results of luciferase assays after transient transfection of the indicated plasmids into HD11 cells are diagramed. GaIRE, Gal4-responsive element; +, the Gal-LZ construct cotransfected with either c-Fos or c-Jun expression vectors as indicated. Gal-JunTAD is a fusion protein consisting of the Gal4 DNA-binding domain and the Jun trans-activation region. Bars, deviation from the mean of duplicate samples.

Fig. 3. The transcriptional activity of different Jun-related (A) and Fos-related (B) AP-1 proteins measured by the Gal4 adaptor assay. GalIRE, Gal4-responsive element; +, Gal-FosLZ (A) or Gal-JunLZ (B) construct cotransfected with the indicated Jun or Fos expression vectors. Bars, deviation from the mean of duplicate samples.

The presence of GalREs does not cause activation of the luciferase reporter gene when transfected into chicken or human tissue culture cells (Column 1, hatched box; and data not shown). Cotransfection of the 5x GalIRE reporter with Gal-JunLZ, Gal-FosLZ, or constructs expressing c-Jun or c-Fos does not affect luciferase levels either (Columns 2, 3, 4, and 5, hatched boxes), whereas a fusion protein consisting of the Gal4 DNA-binding domain and the trans-activation domain of c-Jun causes an 8-fold activation (Lane 10). However, when Gal-JunLZ is transfected together with c-Fos or Gal-FosLZ together with c-Jun, a strong activation of the Gal4-responsive reporter construct is elicited (Columns 6 and 9, hatched boxes). The response is binding site dependent, as a reporter lacking GalREs remains silent with these combinations of expression constructs (Fig. 2, solid boxes). These results support the idea that transcriptional activation is only achieved when the reporter gene promoter is in a ternary complex with the Gal4 adaptor molecule and c-Jun or c-Fos. Consequently, c-Jun and c-Fos do not need to be bound directly to DNA to activate transcription, and, consistent with this interpretation, a point mutant of c-Jun that is unable to bind to DNA shows the same activity as the wild-type protein (data not shown).

Moreover, reporter activation was not observed with the other two possible combinations, namely Gal-FosLZ with c-Fos and Gal-JunLZ with c-Jun (Fig. 2, Columns 7 and 8, hatched boxes). This implies that homodimer-type interactions between two c-Fos or two c-Jun leucine zippers are not stable enough to hold a ternary complex together and thus fail to elicit transcriptional activation in this system. The inability of homologous leucine zippers to dimerize in vivo also precludes an interaction of the adaptor molecules with themselves, which would possibly complicate the interpretation of the assay.

Functional Comparison of Different Jun and Fos Proteins. The Gal4 adaptor assay outlined above facilitates an analysis of the transcriptional activity of individual AP-1 family members generated from conventional expression vectors. This contrasts with previous strategies which have relied on the synthesis of fusion proteins between the transcriptional activator of interest and a heterologous DNA-binding domain, as in the case of the direct Gal4-JunTAD fusion (Fig. 2, Lane 10). This is often tedious and may impair the normal function of the fused protein. Following this approach, we repeated the experiment shown in Fig. 2 with expression vectors directing the synthesis of c-Jun, JunB, JunD, c-Fos, and FosB. These experiments were carried out with luciferase reporter plasmids carrying 1X and 5X GalREs. Cotransfection of c-Jun and JunD with the matching Gal-FosLZ adaptor and either reporter (Fig. 3A) results in a strong, up to 8-
fold stimulation of transcription with the 5× GalRE construct and slightly less with the 1× construct. JunB elicits a weaker response; it generates only a 4–6-fold activation of the 5× GalRE reporter, whereas no significant activation of the 1× GalRE reporter was detected. This observation is in agreement with the data of Chiu et al. (14), who observed a similar activation behavior of JunB toward one or more AP-1 binding sites. Experimental conditions were chosen such that overexpressed Jun and Fos proteins were present in excess over the other components of the assay (adaptors and reporters) so that variations in their concentrations had little influence on induction levels (data not shown). As judged by Western blot with cross-reactive antibodies, all Jun proteins are overexpressed to significant levels and migrate at their expected molecular weights (data not shown).

A complementary experiment was then performed to examine the activity of c-Fos and FosB. Again, the 1× and 5× GalRE reporters were used, but this time cotransfected with the Gal-JunLZ adaptor molecule. c-Fos and FosB elicited similar levels of activation, as shown in Fig. 3B. We also included point mutants of Fos and Jun in these experiments that possess dimerization-deficient leucine zippers (Fig. 3A, JunMuti23, and 3B, MutFos). These proteins do not produce significant levels of activation.

Our comparative studies show that the contribution of the individual Fos and Jun family members to the transcriptional activity of AP-1 can differ. Such differences may serve as one explanation for the existence and evolutionary conservation of families of basically similar transcription factors.

**Analysis of Zipper-Zipper Interactions in Vivo: Zipper Mutants.** The Gal4 adaptor system requires the stable interaction of two leucine zippers in the transfected cell, one on the adaptor and one on the protein that supplies the transcriptional activation domain. A further application of the assay system would be to analyze the dimerization ability of a defined pair of leucine zippers in vivo. This can be achieved by using different combinations of adaptor- and/or zipper-containing transcription factor constructions.

We analyzed a series of the c-Jun proteins with mutations in the leucine zipper that show interesting phenotypes when analyzed in vitro. Mut17 and Mut18 are mutations in c-Jun in which two amino acids, COOH-terminal of leucines 2 and 3 of the zipper, respectively, are changed to isoleucine and aspartic acid (as shown in Fig. 4A). c-Jun proteins carrying these mutations have an enhanced ability to homodimerize, resulting in more stable DNA binding and consequently a higher transcriptional potential in vitro (26). Mutations with a similar phenotype have also been documented and studied by others (18, 27). In a cell, increased c-Jun activity can lead to transformation, and in the organism, to cancer (28, 29). Therefore, it seems surprising that apparent up-mutants of the protooncogene product c-Jun like Mut17 and Mut18 can be encountered so frequently in in vitro experiments.

To investigate this issue, we constructed GalMut17JunLZ and Gal-Mut18JunLZ adaptor proteins, which are the mutant counterparts of the wild-type Gal-JunLZ. These plasmids were cotransfected with vectors expressing either wild-type c-Jun or variants harboring Mut17 and Mut18 (JunMut17 and JunMut18, respectively). As a negative control, we included JunMut23. This carries a leucine to proline substitution at position 3 of the zipper which precludes dimer formation (26). The activity of these Jun derivatives in combination with the different adaptors was compared to that of wild-type c-Fos in the same assay. The results are shown in Fig. 4B. Mutation Mut17, when present in both proteins in the assay, results in a modest, 2-fold, activation of the reporter gene, indicating an enhanced ability of this mutant to homodimerize compared to wild-type c-Jun (Fig. 4B, compare Columns 4 and 8). Such an effect is not observed with Mut18 (Column 11), presumably reflecting the weaker “up-phenotype” of this mutation seen in vitro (26). The analysis of the protein mutants was then extended to their ability to dimerize with a c-Fos leucine zipper. The Gal-JunMut18LZ adaptor molecule appears to possess an activity comparable to the original Gal-JunLZ with respect to heterodimerization with c-Fos (compare Columns 16 and 18). Interestingly, the adaptor molecule carrying Mut17 (Gal-JunMut17LZ), which exhibited an increased homodimerization potential, is severely compromised in its ability to interact with c-Fos in vivo (compare Columns 16 and 17). Apparently, the increased homodimerization potential of the c-Jun mut- tant correlates with a decreased ability to form heterodimers with c-Fos. This finding offers an explanation for the dilemma outlined above: even though Mut17 results in an up-phenotype for homodimerization, it impairs the formation of the predominantly active form of AP-1, the Fos/Jun heterodimer. Therefore, a mutation like Mut17, should it arise spontaneously within the cell, would lead to an overall decrease of Jun activity, with no danger of creating a hyperactive, possibly transforming version of the transcription factor.

**Discussion**

In this paper, we use the Gal4 adaptor assay to study the function of leucine zipper-containing transcription factors. The fact that this assay works underlines the remarkable flexibility of eukaryotic transcription factors in terms of their position relative to the basic transcription machinery. Moreover, it demonstrates that the DNA-binding and transcriptional activation functions of gene-selective transcription factors are, at least in the cases investigated, completely separable and that these proteins can activate transcription even when not bound directly to DNA.

**Analysis of Zipper-Zipper Interactions.** A significant result of our analysis is the observation that ternary complexes depend on heterotypic interactions, i.e., between two Jun or two Fos leucine zippers, cannot be detected; however, heterotypic interactions mediated by a Fos and a Jun zipper occur. Although these results may not be directly comparable to the normal situation in which native AP-1 dimers form and both protomers interact with DNA, they indicate that Jun-Jun and Fos-Fos dimerization is significantly less stable in the cell than Fos-Jun heterodimerization, confirming similar results obtained in vitro (18). This is in line with the concept of mutually dependent binary transcription factors, outlined in the “Introduction.” Thus, we were especially interested to study a particular class of Jun mutant proteins that was described previously by several independent laboratories. In vitro, these mutants showed an enhanced ability
to homodimerize and, consequently, a more stable association with their DNA binding site. The Gal4 adaptor assay was used to analyze interactions between defined pairs of such dimerization mutants of Jun in vivo. Whereas (at least in the case of the more severe mutant Mut17) an enhanced ability to homodimerize was observed in accordance with the in vitro data, the stability of interactions with c-Fos was severely compromised. Hence, it would appear that the sequence of the c-Jun leucine zipper contains residues (like those altered by Mut17) that prevent homotypic interactions in favor of heterotypic ones with, for example, c-Fos-like molecules. Such a preference is an expected property of the "binary transcription factor system" discussed above, and it increases selectivity of complex formation in vivo.

**Assay of Interactions between Members of the BZip Class of Proteins.** The Gal4 adaptor assay should be especially useful for the rapid determination of potential complex formation between already identified bZip proteins of various families and novel candidate proteins of this group. In this way, we have obtained results indicating that proteins of the AP-1 class cannot interact with those of the C/EBP class of bZip proteins in vivo (data not shown).

**Assay of the Transcriptional Activity of AP-1 Factors.** Under the experimental conditions used in this paper, the transfected Gal4 adaptor proteins were in excess over endogenous AP-1 factors, which are not abundant in the cell lines used here. Consequently, reporter gene activity seen after cotransfection of the adaptor alone was low and allowed comparison of the transcriptional activity of individual cotransfected AP-1 proteins, independent of the requirement for a dimerizing partner molecule. For this study, we chose three Jun- and two Fos-like proteins.

The results that we obtained are consistent with and extend previous transfection studies in which the activity of Fos and Jun proteins are measured as dimers binding to AP-1 promoter elements in F9 (14) and NIH3T3 cells (30, 31). In our assay, the activities of c-Jun and JunD are comparable and severalfold higher than that of JunB, the weakest activator of the Jun family, c-Fos and FosB ap-

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* A. Leutz and S. E. Smith, unpublished observations.
pear to have an equivalent ability to activate gene expression in this system. Thus, both Jun- and Fos-related factors can activate transcription independently of one another to different degrees. However, the fact that the AP-1 gene family is evolutionarily conserved in all tested vertebrate species belies the importance of the existence of various family members. Additional differences must exist that have not been addressed in this study. Such variations between individual AP-1 factors have indeed been described at the level of spatial (32) and temporal expression (33). Furthermore, it appears that their pattern of induction can depend on the nature of the extracellular signal (34). We plan to use the Gal4 adaptor approach described here to analyze the roles that individual AP-1 protomers play in these complex signal transduction functions.

Materials and Methods

Plasmid Constructions

All protocols for the molecular cloning techniques can be found in Ref. 35.

Reporter Plasmids. The minimal promoter of the hMTII\(\alpha\) gene, obtained from the MCAT plasmid previously described (26), was cloned in front of the luciferase gene in the plasmid X-P1 (36) to generate 8x GalRE. The Gal4 luciferase reporters were generated by inserting oligonucleotides containing Gal4 DNA binding sites immediately upstream of the hMTII\(\alpha\) promoter. The sequences of the sites are: Gal4: 5' - AGCGGAGTACTGTCCCTCGAG-3'.

Gal4-luciferase reporters possessed either one or five Gal4 binding sites (1x GalRE and 5x GalRE, respectively).

The RSV-\(\beta\)galactosidase expression vector has been described previously (37).

The Gal4-Leucine Zipper Expression Plasmids. All expression vectors used in this study contain the RSV long terminal repeat and the SV40 polyadenylation signal. Hybrid genes encoding leucine zippers derived from human c-Jun (codons 266–327; Gal-JunLZ), rat c-Fos (codons 156–202; Gal-FosLZ), or various mutants (Gal-JunMut17LZ, Gal-JunMut18LZ, and Gal-JunMut23LZ) (26) were synthesized by polymerase chain reaction and inserted in frame with a DNA fragment from the Gal4 gene encoding the DNA-binding domain (codons 1–147) into pCM4, an RSV long terminal repeat-SV40 polyA eukaryotic expression vector.\(^5\)

Jun and Fos Expression Vectors. c-Jun and c-Fos vectors expressing human and rat genes, respectively, have been described elsewhere (38). The leucine zipper mutations Mut17, Mut18, and Mut23 (26) were introduced into c-Jun to create JunMut17, JunMut18, and JunMut23. MutFos represents a mutant of v-Fos which is defective in its ability to dimerize with Jun and is expressed from the plasmid E300-L3L4L5 described in Ref. 39.

RSV-driven JunB and JunD expression vectors were gifts from M. Yaniv and have been described previously (31). The FosB gene was obtained from M. Zerial (40) and cloned into the RSV-SV40 polyA expression vector (FosB).

Gal-JunTAD was generated by fusing codons 1–268 of human c-Jun 3' to the Gal4 DNA-binding domain and expressing this chimera from a RSV-based expression vector.

All constructions were verified by restriction digest and sequencing.

Cells and Tissue Culture

The macrophage cell line HD11 (also called HCB1) is described in Ref. 41. These cells were cultured in 5% CO\(_2\) at 37°C in Eagle's medium supplemented with 8% fetal calf serum, 2% heat-treated chicken serum, and 10 mM HEPES-NaOH (pH 7.4).

Transfections and Trans-activation Assays

Chicken HD11 cells were transfected using a modification of published procedures (42). In brief, HD11 cells were trypsinized and plated in fresh growth medium to a density of 1 × 10\(^6\)/6-cm dish. The cells were grown overnight, and the monolayers were rinsed twice with MEM supplemented with 25 mM HEPES-NaOH (pH 7.4). The DNA samples were added in 1 ml of HEPES-MEM containing 0.5 mg/ml DEAE-dextran. The dishes were left at room temperature and then rinsed once with HEPES-MEM before fresh medium was added, and they were returned to the 39°C, 5% CO\(_2\) incubator. Typically, 1 µg of the various reporter constructs was cotransfected with 0.5 µg of each of the effector plasmids (either Gal4-LZ constructs alone or together with 0.5 µg of the c-Jun or c-Fos expression vectors). RSV-\(\beta\)-galactosidase (0.3 µg) was included in each dish as an internal control of transfection efficiency, and the total quantity of DNA transfected per dish was kept constant at 2.3 µg by addition of pUC18. After 24 h, the monolayers were rinsed with cold phosphate-buffered saline, and the cells were scraped into 1 ml of cold phosphate-buffered saline, transferred into a microcentrifuge tube, pelleted, and placed on ice. 1% Triton X-100 in 0.1 M potassium phosphate buffer (pH 7.8) was used to lyse the cells. Published procedures were used for luciferase (43) and \(\beta\)-galactosidase (37) assays. All transfections were performed in duplicate and repeated two or more times with essentially identical results. Luciferase assay results were normalized to the \(\beta\)-galactosidase activity.

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References


\(^5\) E. DeSimone, unpublished.


