Korean J Biol Sci 1: 647-651, 1997

A Proline- and Leucine-rich 19 Amino Acid Oligopeptide from FS1 Functions as a Transcriptional Repression Domain

Yong-Seok Cho, Gumhee Baek, Sangsoon Yoon, Donguck Han, and Kyuhyung Han*

Department of Genetic Engineering, Hallym University, Chunchon 200-702, Korea

Key Words:
Transcriptional repression
Drosophila melanogaster
S2 cell

We have used a transient expression assay employing *Drosophila* S2 cells to study the transcriptional repression activity of a 27 amino acid residue-long repression domain FS1 which was generated by a frame-shift in a pair-rule gene, *even-skipped* of *Drosophila melanogaster*. In an attempt to define a minimal requirement for the repression activity, we constructed a series of truncation mutant forms of the FS1, fused to a heterologous GAL4 DNA-binding domain, and measured their activities. All of the mutant forms, including the GAL4-FS1 (5-23) which retains the smallest number (19) of amino acid residues of FS1, were found to repress an initiator, a minimal TATA-lacking promoter, in a GAL4-binding-site-dependent manner. These findings suggest that a 19 amino acid residue-long region, rich in proline and leucine residues, is a transcriptional repression domain and may interact with the general transcription machinery.

Precise regulation of gene expression is an essential prerequisite for an organism to respond properly to changing internal and external conditions. Regulation of gene expression is primarily achieved at the transcriptional initiation level through interactions among a number of transcription factors, which include sequence-specific and general transcription factors. Sequence-specific transcription factors can be classified into two groups: transcriptional activators and repressors. Several mechanisms have been proposed for the mode of activities of transcriptional repressors (Levine and Manley, 1989; Jackson, 1991).

One of several types of eukaryotic transcriptional repressors are direct repressors that are believed to interfere with the formation or activity of the general transcription machinery (Levine and Manley, 1989; Hanna-Rose and Hansen, 1996). Direct repressors, like transcriptional activators, consist of a modular structure. Therefore, both direct repressors and activator proteins contain a sequence-specific DNA-binding domain and effector domain(s), which is either an activation domain or a repression domain for the activator and the repressor proteins, respectively. The repression domains may interact with a part of the general transcription machinery directly (Um et al., 1995), or indirectly through corepressors. A great number of cases for the interaction between transcription repressors and corepressor have been reported recently (*e.g.*, Friedman et al., 1996; Tong et al., 1996).

* To whom correspondence should be addressed.
Tel: 82-361-240-1467, Fax: 82-361-56-3420

Even-skipped (Eve; Frasch et al., 1987) protein of *Drosophila melanogaster* is a direct repressor. Using transient expression assays, Eve was shown to repress transcription from a minimal basal promoter containing Eve-binding sites upstream of the promoter (Han and Manley, 1993). This repression activity was observed *in vitro* as well (Biggin and Tjian, 1989), and evidence was provided that Eve can interfere with transcription complex assembly *in vitro* (Johnson and Krasnow, 1992). Moreover, Eve was shown to interact physically with the TATA-binding protein (TBP), one of the general transcription factors (Um et al., 1995).

In the course of studying the *Drosophila* Eve direct repressor protein, an artificial 27 amino acid residue-long repression domain FS1, which was produced by frame-shift, was identified (Han and Manley, 1993). The FS1, when fused to a heterologous GAL4 DNA-binding domain or to the homeodomain of Eve transcriptional repressor protein, was previously shown to repress transcriptions driven by transcriptional activators Sp1 and GAL4-VP16, respectively (Han and Manley, 1993). Furthermore, the Eve-ABFS1 protein was shown to interact physically with TATA-binding protein (TBP) *in vitro* (Um et al., 1995). These findings suggest that FS1 is a part of a direct repression domain which binds to the general transcription machinery.

In this study, we examined whether the FS1 by itself is sufficient to act as a direct repression domain and attempted to define a minimal repression domain by constructing truncated forms of the FS1 and measuring their activities using transient transfection assays.

Materials and Methods

Recombinant plasmids

The *in vivo* expression vector pAct5CPPA, pActGAL4, and GAL4-FS1 expression plasmid were described previously (Han et al., 1989; Han and Manley, 1993). The following recombinant plasmids were constructed by standard subcloning procedures. Expression plasmids for the truncation mutants of GAL4-FS1 {GAL4-FS1 (2-23), GAL4-FS1 (5-27), and GAL4-FS1 (5-23)} were constructed from pActGAL4-FS1 (2-27), exploiting *Sph*I and *Msc*I restriction sites. Sequences around the junctions of the fusion proteins were confirmed by DNA sequencing. The reporter plasmid pGSI-luc was constructed from pGSI-CAT (Han and Manley, 1993) and pluc (Cho et al., 1996). The pTATA-luc was constructed from pTATA-CAT (Han, unpublished) and pluc. Detailed strategies used for constructing recombinant plasmids are available upon request.

DNA transfection and transient expression assay

DDAB-mediated transfection of *Drosophila* Schneider Line 2 cells (S2 cells) was performed using 24-well tissue culture plates as described previously (Han, 1996). Each transfection mixture contained 10 ng of expression plasmid, 10 ng of reporter plasmid, and 100 ng of pcopiaLTR-lacZ internal control plasmid. Three days after transfection, cell extracts were prepared by adding 20 µl of CLR (cell lysis reagent, Promega) to each well after aspiration. After centrifugation, two µl and one µl of the supernatant were taken for β-galactosidase assay and luciferase assay, respectively. The β-galactosidase assay was performed by a colorimetric method as described previously (Han and Manley, 1993). The luciferase assay was performed with a luminometer (TD-20/20, Turner Designs) essentially according to the method described by Fulton and Ness (1993). Normalized luciferase activities were calculated by determining the luciferase/β-galactosidase activity ratios and averaging the values from duplicate experiments. Each transfection was repeated several times (2 to 20 times) and the average values (±SD) were presented.

Gel-mobility shift assay

Nuclear extracts were prepared by scaling up the transfections essentially as described previously (Han and Manley, 1993) with following modifications. Sixty-mm tissue culture dishes were used and each transfection contained 600 ng of the GAL4 fusion expression plasmid, 400 ng of pGem1, and 1000 ng of pcopia LTR-lacZ as an internal control. Transfection efficiencies were determined by assaying for β-galactosidase activities as described above. The DNA used in the gel mobility shift analysis was a 0.11 kb 5GAL4 (Fischer et al., 1988) *Hind*III-*Xba*I fragment end-labeled with Klenow fragment and DIG-11-dUTP (Boehringer Mannheim). Binding reactions were performed in a volume of 10 µl in 20 mM HEPES (pH 7.8), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 2.5% CHAPS, and 10% glycerol and were incubated for 20 min on ice. Reaction mixtures contained 1 µl of nuclear extract, 1 µg of poly[d(I-C)], 1 µg of poly[d(A-T)], 2 µg of sonicated salmon sperm DNA, and 2 ng of labeled 5GAL4 DNA. Samples were loaded on a prerun 4% polyacrylamide gel (80:1, acrylamide/bis) containing 0.5 X Tris-Glycine-EDTA buffer. Electrophoresis was at 120 V at room temperature for 1-2 h. After electrophoretic separation, the probe DNA was transferred to a nylon membrane (Hybond-N$^+$, Amersham) by electroblotting and baked at 80℃ for 2 h. The probe DNA on nylon membrane was detected by using anti-digoxigenin-POD Ab (Sigma) and ECL reagents (Amersham).

Results

GAL4-FS1 fusion protein represses a basal promoter

FS1, when fused to a heterologous GAL4 DNA-binding domain, was previously shown to repress transcriptions stimulated by a transcriptional activator Sp1 (Han and Manley, 1993). Furthermore, FS1 was required for the Eve-ABFS1 protein, a fusion protein between amino-terminal region of Eve and FS1, to interact physically with TATA-binding protein (TBP) *in vitro* (Um et al., 1995). These findings suggest that FS1 is at least a part of a direct repression domain which can bind to

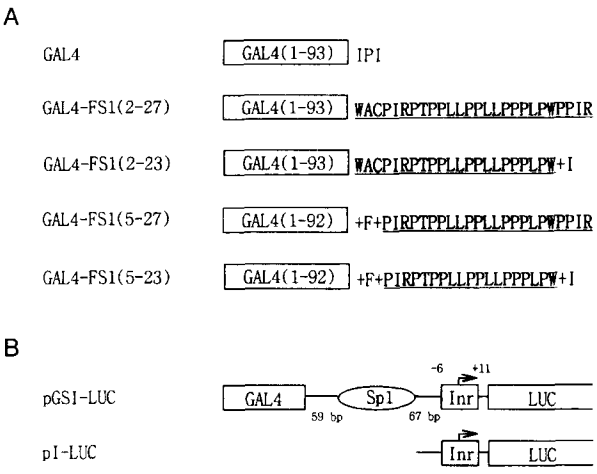

Fig. 1. A series of GAL4-FS1 truncation mutants and reporter plasmids used in this study. A. GAL4-FS1 fusion proteins. Coding regions for the GAL4 DNA-binding domain (1 to 92 or 93) with or without FS1 fusions were inserted downstream of a constitutive actin 5C gene promoter (Han et al., 1989). The numbers in parentheses indicate amino acid residues from the N-terminus of FS1 or GAL4 DNA-binding domain. The primary structures of each oligopeptide fused to GAL4 DNA-binding domain are shown with the single-letter amino acid code and residues of FS1 are underlined. B. Luciferase reporter plasmids. The pI-LUC reporter plasmid contains the TdT initiator (Inr), a TATA-less basal promoter, upstream of the firefly luciferase cDNA (LUC). The pGSI-LUC reporter plasmid contains synthetic GAL4 binding sites and GC boxes for Sp1 binding upstream of the initiator.

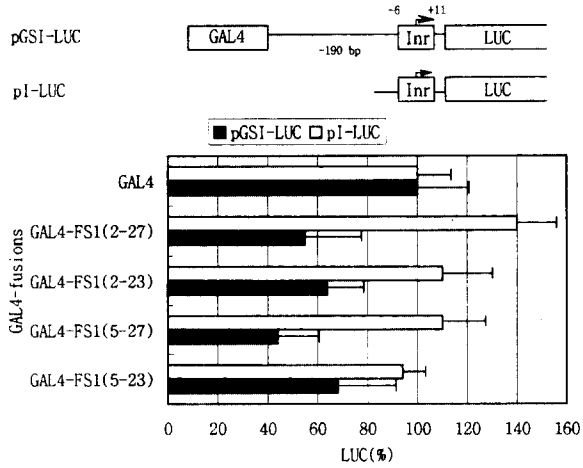

Fig. 2. GAL4-FS1 fusion proteins repress a basal promoter. Ten ng each of the basal promoter-LUC reporter plasmids, with or without GAL4 binding sites, as diagramed above the graph, was cotransfected into *Drosophila* S2 cells with 10 ng each of the GAL4-FS1 fusion protein expression plasmids and 100 ng of an internal control pcopiaLTR-lacZ plasmid. Relative Luciferase activities (±SD) are shown in which the percent activity of GAL4 DNA-binding domain without FS1 fusion was taken as 100%.

general transcription machinery. To determine whether FS1 itself is a direct repression domain, we asked whether GAL4-FS1 fusion proteins could repress expression from a plasmid containing only a single core promoter element, the 17-bp TdT initiator (Smale and Baltimore, 1989), without any transcriptional activator bound on the plasmid. For this, a GAL4-FS1 (2-27) expression plasmid and reporter plasmids (pGSI-luc, pI-luc; Fig. 1B) containing the TdT initiator upstream of the firefly luciferase gene, plus or minus GAL4-binding sites (Fischer et al., 1988) ~190 bp upstream of the initiator, were introduced into *Drosophila* S2 cells by transient cotransfection as described previously (Han et al., 1989; Han, 1996). Fig. 2 shows that GAL4-FS1 (2-27), which contains from the second to the twenty-seventh amino acid residues of FS1, repressed basal promoter activity, albeit weakly, whereas it did not affect promoter activity significantly in the absence of GAL4-binding sites. These results strongly suggest that the FS1 is a direct repression domain.

Central 19 amino acid residues of the FS1 retains repression activity

In an attempt to define a minimal repression domain in FS1, a series of truncation mutants were generated (Fig. 1A). Expression plasmids for an N-terminal truncated GAL4-FS1 (2-23), a C-terminal truncated GAL4-FS1 (5-27), and an N,C-truncated GAL4-FS1 (5-23) were constructed and tested for their repression activities. In a first set of experiments, we asked whether these truncated proteins could repress a basal promoter to determine minimal requirements for a direct repression domain. The pGSI-luc or pI-luc was cotransfected with each of the GAL4-FS1 expression

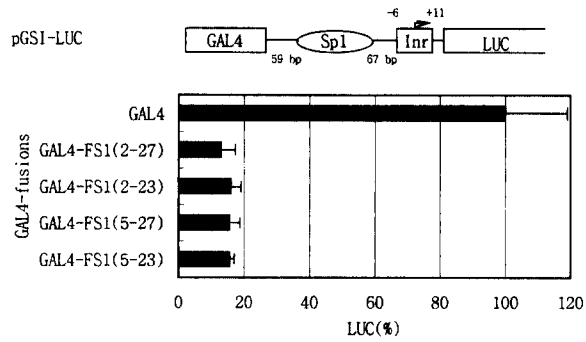

Fig. 3. GAL4-FS1 fusion proteins repress activated transcription strongly. Ten ng of the pGSI-LUC, diagramed above the graph, was cotransfected with 10 ng each of the GAL4-FS1 fusion protein expression plasmids, 0.1 ng of Sp1 transcriptional activator expression plasmid (pAct-Sp1; Han and Manley, 1993), and 100 ng of an internal control pcopiaLTR-lacZ plasmid. Relative luciferase activities (±SD) are shown in which the percent activity of GAL4 DNA-binding domain without FS1 fusion was taken as 100%.

plasmids. Fig. 2 shows that all three truncation mutants repressed luciferase expression, albeit weakly, when GAL4-binding sites were located upstream of the initiator, whereas it did not affect promoter activity significantly in the absence of GAL4-binding sites. These results suggest that the central 19 amino acid residues, from fifth to twenty-third amino acid residues of FS1, can function as a direct repression domain.

Since the repression activities observed above were not very impressive, we wanted to confirm the repression activities by taking advantage of the fact that GAL4-FS1 repressed transcription stimulated by an activator Sp1 more strongly. For this, reporter plasmids (pGSI-luc), containing GAL4-binding sites 59 bp upstream of Sp1-binding sites (GC boxes; Courey and Tjian, 1988), were cotransfected with each of the GAL4-FS1 expression plasmids and an Sp1 expression plasmid. Fig. 3 shows that all three truncation mutants repressed luciferase expression effectively and as strongly as the full-length FS1 (2-27). Since the GAL4-binding sites were upstream of the Sp1-binding sites, repression was unlikely to be due simply to a physical block preventing interaction between Sp1 and the basal transcription machinery. This result indicates that the central 19 amino acid residues are sufficient to repress activated transcription as well.

All of the GAL4-FS1 fusion proteins bind to the target DNA

To compare the strength of repression activities of the FS1 mutant forms more accurately, we estimated the relative DNA-binding affinities of the GAL4-FS1 (2-27) and mutant proteins by performing gel-mobility shift assays with nuclear extracts prepared from the transfected cells (see Materials and Methods). The DNA probe was an end-labeled ~0.11-kb 5GAL4 fragment isolated from the pGSI-luc plasmid. The results are shown in Fig. 4 and the three truncated

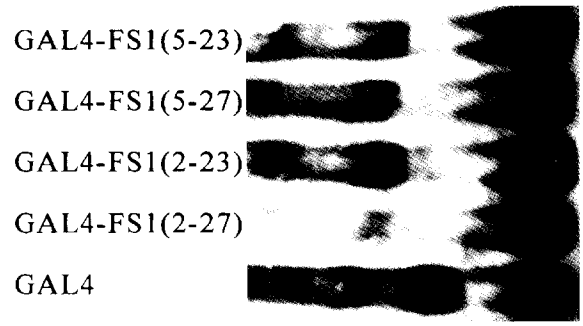

Fig. 4. DNA-binding activities of GAL4 fusion proteins isolated from transfected cells. Transfections and nuclear extract preparation were performed as described in Materials and Methods. Relative transfection efficiencies were estimated by assaying for β-galactosidase activities, and there were no significant differences. Reaction mixtures contained 1 μl of nuclear extract in a volume of 10 μl, and the DNA probe was a 0.11 kb 5GAL4 fragment end-labeled nonradioactively. Conditions for the gel mobility shift assay were as described in Materials and Methods.

FS1 proteins showed similar DNA-binding affinities as the GAL4 DNA-binding domain, whereas the GAL4-FS1 (2-27) showed somewhat lower binding affinity. It is not straightforward to explain why the GAL4-FS1 (2-27) binds to the target DNA weakly, although it is still possible that the GAL4-FS1 (2-27) binds to the target DNA as strongly as other GAL4 fusion proteins when purified proteins are used for the gel-mobility shift assay. The concentration of free GAL4-FS1 (2-27) in nuclear extract might have been reduced due to a specific interaction between FS1 (2-27) and some component(s) of the nuclear extract, resulting in masked GAL4 DNA-binding domain. It is also possible that the association rates for the binding of GAL4 fusion proteins to the target DNA are similar, while the dissociation rate for the GAL4-FS1 (2-27) in a nuclear extract is higher than those for other GAL4 fusion proteins. Although some uncertainties remain, it is clear that all four GAL4 fusion proteins are able to bind to the target DNA. Taken together, these results suggest that the central 19 amino acids of the FS1 are sufficient for repression activity.

Discussion

The FS1 is composed of eight different amino acids (proline, leucine, isoleucine, arginine, threonine, tryptophan, alanine, and cysteine). However, the 19 amino acid residue-long repression domain FS1 (5-23), identified in this study, is composed of 6 amino acids (proline, leucine, isoleucine, arginine, threonine, and tryptophan). This suggests that alanine and cysteine residues, which are components of the FS1, are not required for FS1 to repress transcription.

The 19 amino acid residue-long repression domain FS1 (5-23) is strikingly rich in proline residues (10 out of 19; 53%), and moderately rich in leucine residues (5 out of 19; 26%). The remaining 4 residues are isoleucine, arginine, threonine, and tryptophan (Fig. 1A). Such proline-rich repression domains have been identified from several transcriptional repressors of diverse organisms. For example, a mammalian Evi-1 proto-oncogene product contains a proline-rich repression domain (Bartholomew et al., 1997), a human homeodomain protein EVX1's 51 amino acid residue-long repression domain is rich in alanine and proline residues (Briata et al., 1997), the human Cut homeodomain protein's repression domain is rich in alanine and proline residues as well (Mailly et al., 1996), the repression domain C2D2 of *Drosophila* Even-skipped protein is rich in proline residues (13 out of 57; Han and Manley, 1993), and the yeast Mig1 repressor's repression domain contains several leucine-proline dipeptide repeats (Ostling et al., 1996). However, the FS1 (5-23) is the shortest among such proline-rich repression domains.

How does the proline-rich repression domains work? They may interact physically with a part of general transcription machinery such as TATA-binding protein (TBP). A repression domain FS1 was required for the Eve-ABFS1 protein, a fusion protein between amino-terminal region of Eve and FS1, to interact physically with TBP *in vitro* (Um et al., 1995), raising the possibility that FS1 plays some role in binding with TBP. Furthermore, a human transcription enhancer factor-1 (TEF-1) repressor contains a proline-rich domain which was shown to be essential for TBP binding (Jiang and Eberhardt, 1996). On the other hand, some proline-rich repression domains may interact with corepressors. For example, the leucine, proline-rich repression domain of yeast Mig1 repressor is required for interaction with the Cyc8 and Tup1 proteins (Cassart et al., 1997). Whatever the repression mechanism is, we are in a position to assess how important each of the 6 different amino acid residues (P, L, I, R, T, and W) is for the repression activity, since the length of the FS1 (5-23) repression domain is manageably short.

Acknowledgements

This work was supported by a grant (1995 Genetic Engineering Research) to Kyuhyung Han from the Ministry of Education, Korea. The authors thank Doohyun Lee and Sangyoon Han for their technical assistance.

References

Bartholomew C, Kilbey A, Clark AM, and Walker M (1997) The Evi-1 proto-oncogene encodes a transcriptional repressor activity associated with transformation. *Oncogene* 14: 569-577.

Biggin MD and Tjian R (1989) A purified *Drosophila* homeodomain protein represses transcription *in vitro*. *Cell* 58: 433-440

Briata P, Ilengo C, van de Werken R, and Corte G (1997) Mapping of a potent transcriptional repression region of the human homeodomain protein EVX1. *FEBS Lett* 402: 131-135.

Cassart JP, Ostling J, Ronne H, and Vandenhaute J (1997) Comparative analysis in three fungi reveals structurally and functionally conserved regions in the Mig1 repressor. *Mol Gen Genet* 255(1): 9-18.

Cho, Y-S, Han D, Baek G, Park S-P, Yoon S, Lim WK, Kim C-R, Kim HD, Kang HS, and Han K (1996) Versatile luciferase reporter plasmids for transcription studies in diverse eukaryotic cells. *Korean J Zool* 39: 378-386.

Courey AJ and Tjian R (1988) Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine-rich motif. *Cell* 55: 887-898.

Fischer JA, Ginger E, Maniatis T, and Ptashne M (1988) GAL4 activates transcription in *Drosophila*. *Nature* 332: 853-856.

Frasch M, Hoey T, Rushlow C, Doyle H, and Levine M. (1987) Characterization and localization of the *even-skipped* protein of *Drosophila*. *EMBO J* 6: 749-759.

Friedman JR, Fredericks WJ, Jensen DE, Speicher DW, Huang XP, Neilson EG, Rauscher FJ III (1996) AP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes & Dev* 10: 2067-2078.

Fulton R and Ness BV (1993) Luminescent reporter gene assays for luciferase and β-galactosidase using a liquid scintillation counter. *Biotechniques* 14: 762-763.

Han K, Levine MS, and Manley JL (1989) Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell* 56: 573-583.

Han K and Manley JL (1993) Transcriptional repression by the *Drosophila* Even-skipped protein: definition of a minimal repression domain. *Genes & Dev* 7: 491-503.

Han K (1996) An efficient DDAB-mediated transfection of *Drosophila* S2 cells. *Nucleic Acids Res* 24: 4362-4363.

Hanna-Rose W and Hansen U (1996) Active repression mechanisms of eukaryotic transcription repressors. *Trends in Genetics* 12: 229-234.

Jackson ME (1991) Negative regulation of eukaryotic transcription. *J Cell Sci* 100: 1-7.

Jiang SW and Eberhardt NL (1996) TEF-1 transrepression in BeWo cells is mediated through interactions with the TATA-binding protein, TBP. *J Biol Chem* 271: 9510-9518.

Johnson FB and Krasnow MA (1992) Differential regulation of transcription preinitiation complex ssembly by activator and repressor homeodomain proteins. *Genes & Dev* 6: 2177-2189.

Levine M and Manley JL (1989) Transcriptional repression of eukaryotic promoters. *Cell* 59:405-408.

Mailly F, Berube G, Harada R, Mao PL, Philips S, Nepveu A (1996) The human cut homeodomain protein can repress gene expression by two distinct mechanisms: active repression and competition for binding site occupancy. *Mol Cell Biol* 16: 5346-5357.

Ostling J, Carlberg M, Ronne H (1996) Functional domains in the Mig1 repressor. *Mol Cell Biol* 16: 753-761.

Smale S and Baltimore D (1989) The "initiator" as a transcription control element. *Cell* 57: 103-113.

Tong GX, Jeyakumar M, Tanen MR, and Bagchi MK (1996) Transcriptional silencing by unliganded thyroid hormone receptor beta requires a soluble corepressor that interacts with the ligand-binding domain of the receptor. *Mol Cell Biol* 16: 1909-1920

Um M, Li C, and Manley JL (1995) The transcriptional repressor Even-skipped interacts directly with TATA-binding protein. *Mol Cell Biol* 15: 5007-5016.

[Received June 17, 1997; accepted July 30, 1997]