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Active repression of organizer genes by C-terminal domain of PV.1

Yoo-Seok Hwang,[a] Hyun-Shik Lee,[b] Dong-Hyun Roh,[c] Sang-Wook Cha,[a] Sung-Young Lee,[b] Jeong-Jae Seo,[a] Jaebong Kim,[b] and Mae Ja Park[a,*]

[a] *Department of Anatomy, School of Medicine, Kyungpook National University, Taegu 700-422, South Korea*
[b] *Department of Biochemistry, College of Medicine, Hallym University, Chun-Cheon, South Korea*
[c] *Department of Microbiology, Chungbuk National University, Cheoungju 361-763, South Korea*

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Abstract

PV.1, a homeotic protein, ventralizes dorsal mesoderm and inhibits neuralization by mediating *BMP-4* signaling in *Xenopus* embryo. In our previous report antimorphic PV.1 causes a secondary axis by inducing the ectopic organizer. We analyzed the structure of this transcription factor through domain level assessment. In a phenotype-inducing test, half of the N-terminus at the N-terminal side was unessential for inducing ventralization of embryos. We examined the transacting activity of several regions of PV.1 utilizing GAL4 hybrid system. The C-terminal region/GAL4DBD (DNA binding domain) exhibited strong repressive activity on a reporter gene (operator/promoter/reporter; Gal4-TK-luc) as much as the whole polypeptide/GAL4DBD, whereas the N-terminal region/GAL4DBD showed only modest repression. The results suggest that PV.1 functions as a transcriptional repressor and this repressive activity is localized mostly to the C-terminal region. Additional characterizations of N- and C-terminus with respect to the effects on the expression of other genes are described.
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In vertebrate embryogenesis, early patterning is instructed by a precise spatiotemporal gradient of hormone-like signaling molecules. In *Xenopus*, among these, bone morphogenetic protein 4 (BMP-4), a member of the TGF-β superfamily, has been widely known to be the key molecule in dorsoventral patterning of mesoderm and neural-epidermal determination of ectoderm [1,2]. The BMP-4 gradient along the dorsoventral axis develops [3] in antagonism with chordin [4], noggin [5], follistatin [6], Xnr-3 [7], and Cerberus [8,9]. This gradient information is then transferred into the responding cells. At the intracellular level, BMP-4 signal is transduced by Smad proteins and exerts its effect through an increased expression of its target molecules, so called 'ventralizing factors' or 'ventral mediators' which induce actual ventral fate mesoderm or epidermal fate ectoderm. The ventralizing factors have been identified and characterized in the BMP-4 signaling hierarch during the early development of *Xenopus*.

These ventralizing factors can be categorized into several groups: Xvent-1 (includes *Xvent-1* [10], *Xvent-1B* [11], and *PV.1* [12]), Xvent-2 (includes *Xvent-2*, *Xvent-2B* [13], *Xom* [14], and Vox1 (also known as Xbr1b), Vox15 [15], and Xbr1a [16]), GATA (the zinc-finger transcription factors includes *GATA-1a, b* [17], and *GATA-2* [18]) and noncategorized *Xmsx-1* [19], *Xvex-1* [20]. Except for the GATA group, all other genes described above are homeotic genes encoding divergent transcription factors bearing similar homeodomain, but distinguishable N- and C-terminal domain. The diversity, with subtle differences, of these ventralizing factors is thought to be responsible for the variety of fate-determination in germ layers caused by BMP signaling [11]. The gain-of-function and loss-of-function studies for these ventral mediators (*Xvent-1,2* [21], *Vox* [22], *Xvex-1* [23], *Xmsx-1* [24], *GATA-2* [25], and *PV.1* [26]) have presented evidence supporting the idea that the

* Corresponding author. Fax: +82-53-426-9085.
E-mail address: mjpark@knu.ac.kr (M.J. Park).

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antagonism in extra-cellular space reappears at the intracellular level as a reciprocal repression of gene expression between dorsal and ventral transcription factors.

For the majority of these genes, however, the mechanisms of mutual repression between the dorsal and ventral transcription factors are widely unknown in that they may act as direct repressors to each other or, with as much of a possibility, they may also function as indirect transcriptional activators. In addition, the possibility of dual functioning exerting repression to opponents and activation to its partners cannot be excluded. An example of this was introduced by the finding of the dual functioning of Xvent-2 (closely related to Xom) which previously had been proved to be a transcriptional repressor up-regulating *BMP-4* transcription by direct binding to the promoter region of the *BMP-4* gene [27].

To date, all of the ventralizing factors mentioned above except for GATA-2 have been assumed to be transcriptional repressors because the mutant chimeric proteins used in the loss-of-function studies, designed to drive transcriptional activation by fusing VP16 transactivation domain, actually mimic the DNBR (dominant negative BMP-4 receptor) effect. The structures of these proteins, however, have not been studied in a condition free of artificial force driven by substituted transacting domains except for Xom for which the structure/function study made use of the GAL4 hybrid system [28].

PV.1 belongs to the *Xvent-1* group. Our previous study utilizing an antimorphic construct indicates that PV.1 also acts as a mediator of ventroposterior mesoderm formation instructed by BMP-4. In this paper, we assessed PV.1 at domain level to demonstrate the transacting identity among the repressor and activator and to find out the correlation between functions and domains. Several deletion mutants were examined for their phenotype-inducing competence and by utilizing GAL4 system their transacting activities were compared. We then determined their effect on other genes' expression, especially on organizer genes. The results suggest that PV.1 is a transcriptional repressor and this repressing activity is elicited mostly by the C-terminal domain.

Materials and methods

Constructs. For construction of deletion mutants used in (i) morphology test, (ii) RT-PCR, and (iii) XFD-, Nog-promoter luciferase assay, PCR products were enzyme-digested and ligated with *Eco*RI/*Not*I-cut pSP64TEN vector. For construction of effector RNAs used in GAL4 one hybrid assay, these DNA inserts above were ligated to the 3′ end of the yeast G4DBD (amino acids 1–147) coding region in pCMX-GAL4 (new linker subcloned from pSG424; [29]).

The *Hin*dIII (blunted)–*Xba*I fragments from this construct were then subcloned into *Eco*RI (blunted)/*Xba*I gap of pCS2 vector for stable translation. Cutting out *Eco*RI/*Eco*RI fragment of G4DBD-PV1 and re-ligation created an artificial frame shift at G4DBD-to-PV1 linkage resulting in G4DBD-18a.a [10].

PCR-mediated cloning with a genomic DNA template extracted from adult *Xenopus laevis* muscle rendered XFD-luc construction in pGL2-basic vector, which contains BIE and AAE (−253 to +91; [30]). Nog-luc was gained by ligation of full-length 2066 bp fragment (from pSK-N2066; kindly provided by Dr. DING Xiao Yan; *Bam*HI (blunted)/*Eco*RI (blunted)) of noggin genomic DNA flanking region with pGL3-basic (Stratagene) at its *Kpn*I (blunted)/*Hin*dIII (blunted) gap. This 2066 bp fragment bears 1550 bp of 5′ flanking region retaining activin-, Wnt-, and PV.1-responsiveness and also 516 bp 5′ UTR [31].

mRNA injection, explant culture, and gross examination. PV.1 and other mRNAs were transcribed by the Megascript kit (Ambion) with a cap:GTP ratio of 5:1. *X. laevis* embryos were obtained by artificial insemination after induction of females with 500 units of human chorionic gonadotropin (HCG). The four-cell stage embryos were dorsally or ventrally injected at the equatorial region with indicated amounts of synthetic capped mRNAs coding deletion mutants, β-gal, BMP-4, and PV.1, for examining morphology; PV.1, NH-PV1, and HC-PV1 for RT-PCR assay. Developmental stages were designated according to Nieuwkoop and Faber [32]. The injected embryos were then allowed to develop up to 28–29 stages for observation. For RT-PCR analysis, ventral and dorsal marginal zone explants of some embryos were dissected at stage 10–10.5 and cultured to stage 12 or stage 25. Some uninjected embryos were left intact to serve as whole embryo control group at the defined developmental stages.

Analysis of expression by RT-PCR. Four-cell stage embryos were injected with PV.1, NH-PV1, and HC-PV1 mRNAs (125 pg) at their dorsal midline. Dorsal marginal zone tissue was dissected at an early gastrula stage and cultured until the siblings reached stage 11 or stage 25. Total RNA was extracted from cultured explants with TRIzol reagent (Gibco-BRL) following the manufacturer's instructions. One-twentieth of extracted RNA pool from each group was gathered and used for no-RT control. RT-PCR was performed with a Superscript preamplification system (Gibco-BRL). Reverse-transcribed cDNA pool was PCR-assayed by radioactivity of incorporated [α-^{32}P]dCTP for expression of *Wnt8*, *Xhox3*, *EF1a*, and *globin* or by EtBr staining for expression of *Chordin*, *goosecoid*, *Zic3*, and *EF1a*. Primer sets and PCR conditions were applied as described (List of RT-PCR primers of the De Robertis laboratory).

Embryo extraction and luciferase assay. For XFD-luc and Nog-luc luciferase assay, mutant mRNAs (effector), luciferase expression plasmids (reporter) and, as internal control, 10 pg of pRL-TK (Promega) were co-injected dorsally at four-cell stage embryos and harvested at stage 13. As much as 3–7 embryos were gathered in one tube and 3–5 tubes per each experimental group were lysed (50 μl lysis buffer/embryo) and the lysate (100 μl) was determined for luciferase activity. The whole experiment was repeated 3–4 times. For GAL4-TK-luc assay mRNAs coding G4DBD-PV1-deletion derivatives (effector; 250 pg/blastomere) and GAL4-TK-luc (reporter; 25 pg/blastomere) were co-injected together with internal control DNA at the animal pole of two-cell stage embryos. The animal caps of those embryos were dissected at stage 8.5 followed by 4 h-culture in the MMR solution before luminescence measurement. The harvesting procedure for statistics is the same as was done for XFD-luc and Nog-luc.

Results and discussion

The phenotypes induced by PV.1 deletion mutants

In our previous study, a construct (NH-PV1-EnR) in which *Drosophila engrailed* repressor domain replaces

C-terminus of PV.1 still retained a ventralizing effect like wild type. The result that the domain-swapped protein, NH-PV1-EnR, designed to elicit forced transacting repression still exhibits wild type activities implied that this transcription factor may act as a transcriptional repressor [26].

In order to identify and locate the functional domain of PV.1, we generated several deletion constructs (Figs. 1A and B) and injected mRNAs of these into the dorsal or ventral two blastomeres of four-cell stage embryos. Morphologies of the resulted embryos were then examined at stage 28–29. Those embryos injected with *PV.1* mRNA, as shown with *BMP-4* mRNA (Fig. 2H; also see Table 1 [1,2]), were ventralized showing acephaly, microcephaly, and ultimately Bauchstück (Fig. 2A; also see Table 1; mean DAI = 1.31). Injection of 1/2NHC-PV1 or HC-PV1 still exhibited the wild type effect, inducing severely ventralized phenotype (Fig. 2C for 1/2NHC-PV1, E for HC-PV1; also see Table 1; mean DAI = 2.67 and 1.85, respectively). On the other hand, the embryos injected with the other deletion mutants that lack C-terminal arm (NH-PV1, 1/2NH-PV1, N-PV1, and H-PV1) were at most modestly impaired for normal development when compared to *β-galactosidase* injected embryos (Fig. 2B for NH-PV1, D for 1/2NH-PV1, F for H-PV1, G for N-PV1; also see Table 1).

It is also interesting to note that the ventralized phenotypes of HC-PV1-injected embryos, unlike the other two groups described above (Figs. 2A and C), accompanied an apparent gastrulation defect with a high ratio (Fig. 2E; 92% of ventralized embryos with DAI 0–4). The failed-gastrulation event observed here is not due to the overdose since an injection with an even lower dose (60 pg/blstmr) was sufficient to cause this phenotype (87% of ventralized embryos; data not shown), while an injection with any of the other mutant mRNAs even at a higher dose (500 pg/blstmr) did not cause the gastrulation defect. In other words, the gastrulation defect caused by HC-PV1 is rescued by the co-existence of down 1/2N region.

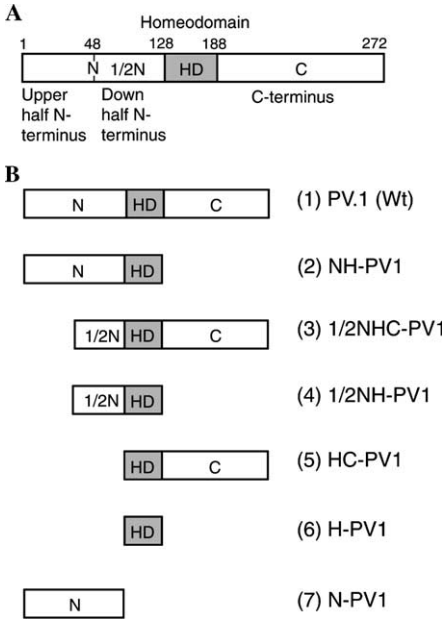

Fig. 1. Schematic diagram of deletion mutants of PV.1 To characterize the functional domain of PV.1, we constructed several deletion mutants and numbered them (1)–(7). (A) Although no region of PV.1 (as indicated; numbers represent amino acids) but homeodomain (HD) shows meaningful amino acid sequence identity in conserved domain database, there are three characteristic regions. N-terminus comprises two distinct regions, the upper half region (upper 1/2N has PHIPCAPQPLPPNKYAKE sequence at its C-terminal side, which includes six prolines within 18 amino acids.) and the other serine-rich acidic down half region (19 serines within 68 amino acids). The whole C-terminal arm is rich in proline (21 prolines within 94 amino acids). (B) Representation of different portions of PV.1 which are encoded by the injected RNAs.

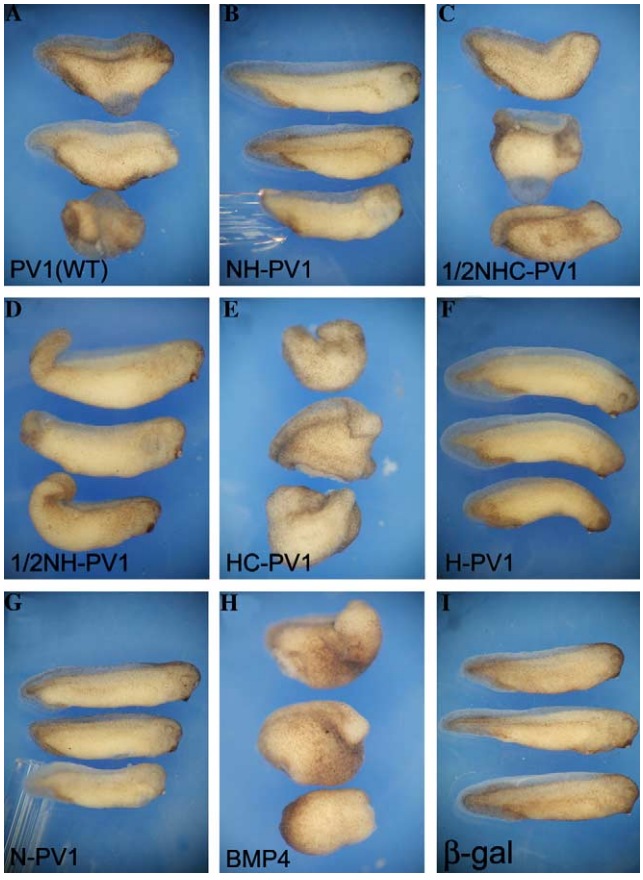

Fig. 2. The phenotype-inducing activities of deletion mutants of PV.1. Four-cell stage embryos were dorsally microinjected (ventral injections had little effects; described only in Table 1) with indicated mRNAs and allowed to grow to stage 28–29. β-*Galactosidase* (0.125 ng/blstmr; same below, otherwise described) injected control (I) embryos were normal. The dorsal injection of *PV.1* (A), 1/2NHC-PV1 (C), and HC-PV1 (E) ventralized embryos like *BMP-4* injection (H) whereas dorsal injection of NH-PV1 (B), 1/2NH-PV1 (D; 0.5 ng/blstmr), H-PV1(F; 0.5 ng/blstmr), and N-PV1(G) did not. (D) and (F) show the small portion exhibiting the most extreme phenotype among resulted embryos (see Table 1).

Table 1
Microinjection of deletion mutants of PV.1

RNA (ng/blstmr)	Site (No. of experiment)	Injected and alive	D A I											Mean
			0	1	2	3	4	5	6	7	8	9	10	
(1) PV1 (WT)														
(0.125)	d (8)	134	**61**	**22**	**23**	**13**	**6**	9						**1.31**
(0.25)	v (3)	26					4	22						4.85
(2) NH-PV1														
(0.125)	d (6)	110				1	4	**95**	**2**	**5**	**3**			**5.14**
(0.25)	v (6)	109						108	1					5.01
(3) 1/2NHC-PV1														
(0.125)	d (4)	101	**1**	**14**	**35**	**27**	**13**	11						2.70
(0.25)	v (1)	21					3	18						4.86
(4) 1/2NH-PV1														
(0.125)	d (2)	50					1	47	2					5.02
(0.5)	d (2)	39			2	1	1	27	4	4				5.08
(0.5)	v (6)	94	1	1	3	1		77	7	4				4.95
(5) HC-PV1														
(0.125)	d (6)	105	**30**	**16**	**23**	**21**	**6**	9						**1.85**
(0.25)	v (1)	17					3	14						4.82
(6) H-PV1														
(0.125)	d (4)	73	1		1	1	2	68						4.84
(0.5)	d (2)	36		3	3	3	1	22	4					4.33
(0.5)	v (4)	98			2	1	7	88						4.85
(7) N-PV1														
(0.125)	d (4)	90		2	1	1	6	78	2					4.81
(0.5)	v (3)	95					2	92	1					4.99
BMP4														
(0.25)	d (2)	38	**10**	**14**	**10**	**2**		1						**1.18**
(0.25)	v (2)	19					3	16						4.84
β-gal														
(0.125)	d (8)	89				1	5	82	1					4.93
(0.5)	v (8)	75					1	73	1					5
	d, dorsal two-cell injection													
	v, ventral two-cell injection		Ventralization				Normal			Dorsalization				

Note. Four-cell stage embryos were dorsally or ventrally injected at the equatorial region with indicated mRNAs followed by incubation until stage 28–29. They were then fixed and scored for DAI. Injection amounts are shown in parentheses. The conspicuous and definitive results for mean DAI are in bold.

We observed here that the N-terminus alone or the homeodomain alone or both together could not ventralize embryos whereas the C-terminus alone with homeodomain was able to ventralize embryos and this combination plus down 1/2N region was sufficient to reproduce exactly the typical PV.1-induced phenotype. This result indicates that if only for the ventralizing effect, the upper 1/2N region is unessential.

PV.1 is a transcriptional repressor and the repression function is localized mostly to the C-terminal arm

The next experiment made use of the GAL4/UAS_G hybrid system to answer the question of transacting identity of PV.1 between repression and activation and to define the minimal transacting domain. We fused various PV.1 deletion mutants to the GAL4 DNA binding domain (G4DBD; Fig. 3A) and compared their transacting activity on UAS_G (operator)/promoter/reporter DNA (Gal4-TK-luc; Fig. 3C).

The basal activity of reporter GAL4-TK-luc markedly enhanced by herpes virus Thymidine Kinase promoter (Fig. 3B). G4DBD-PV1 completely blocked this promoter activity. It is of note that even when compared to the control G4DBD-18a.a, PV.1 strongly repressed the reporter gene (50 times stronger than G4DBD-18a.a). This result provided direct proof of the fact that PV.1 behaves as a transcriptional repressor and this repression is not mediated simply by steric hindrance mechanisms but rather by active repression mechanisms [33].

G4DBD-1/2NHC-PV1 and G4DBD-HC-PV1 also significantly reduced reporter gene activities. More-

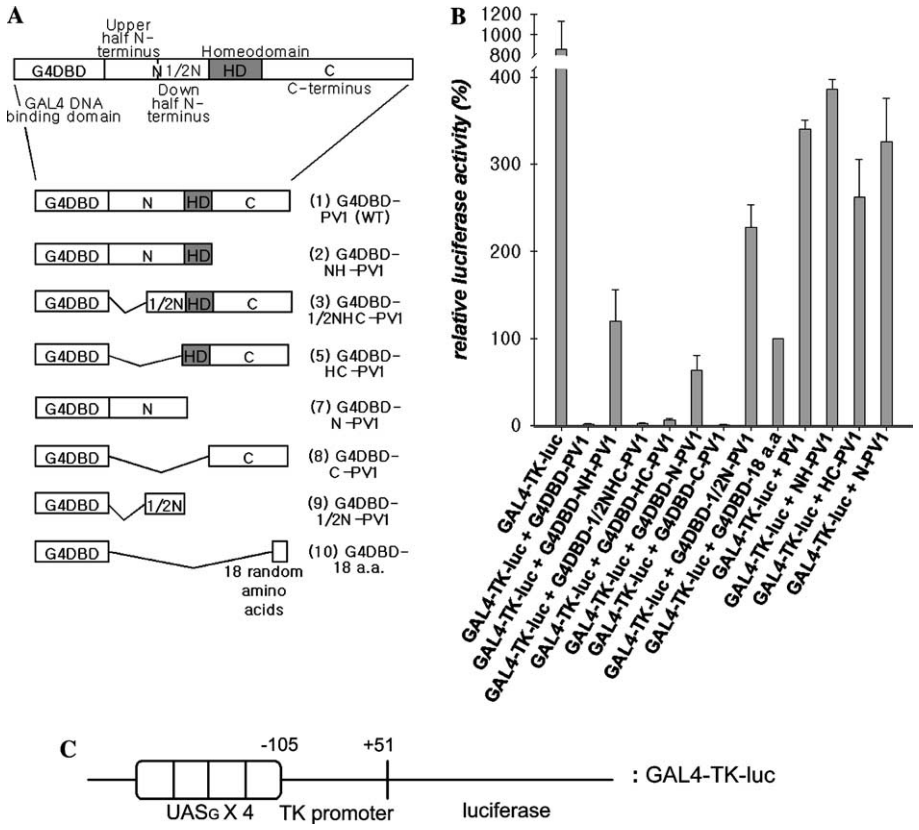

Fig. 3. Schematic diagram of effector constructs and relative luciferase activity of GAL4-TK-luc. (A) We generated fusion proteins by attaching G4DBD (GAL4 DNA binding domain) to deletion constructs of PV.1. We then named and numbered (noted in parentheses) them after the deletion constructs in Fig. 1. G4DBD-18a.a (10) (18 random amino acid-peptide; EFIHSRSALYSPAAPPPYstop) was introduced to count out a probable transacting event imposed by any polypeptide fused to G4DBD and, consequently, to confirm the 'active repression' mechanism. Each effector mRNA (250 pg/blstmr) was co-injected with GAL4-TK-luc (25 pg/blastmr) plasmid DNA at the animal pole of each blastomere of two-cell stage embryos. (C) Four repeated copies of a GAL4-binding upstream activating sequence (UAS_G; 5′-CGACGGAGTACTGTCCTCCGAGCT) were cloned into a TK promoter/luciferase (TK-luc) reporter that contains the herpes virus thymidine kinase promoter (−105/+51) to enhance the basal transcription level of luciferase. (B) At stage 8.5, animal caps were explanted and cultured for four more hours and then harvested for luminescence measurement. Those G4DBD-unfused effector RNAs (PV1, NH-PV1, HC-PV1 and N-PV1) served as controls to count out the nonspecific DNA binding or toxicity of effector proteins. GAL4-TK-luc exhibited highly enhanced reporter gene expression. G4DBD-PV1 (1), G4DBD-1/2NHC-PV1 (3), G4DBD-HC-PV1 (5), and G4DBD-C-PV.1 (8) significantly reduced this enhancement when compared to G4DBD-18a.a while G4DBD-NH-PV1 (2), G4DBD-N-PV1 (7), and G4DBD-1/2N-PV1 (9) did not.

over, these repressions are, respectively, 50 and 16 times as strong as G4DBD-18a.a. Although PV.1 does not have a conserved hexapeptide or an engrailed homology region [34–38], proline-rich feature that is a common characteristic of transcriptional repressors [33] is seen on the C-terminal arm (21 prolines/94 amino acids). The whole C-terminal region hardly can be assumed to contain several repression motifs because these prolines are not clustered but spread evenly on this region.

An observation that G4DBD-C-PV.1 and G4DBD-N-PV1 showed almost twice the level of repression as that of G4DBD-HC-PV1 and G4DBD-NH-PV1 is likely to represent the portion of the contribution of the homeodomain itself to the PV.1 endogenous target, which would otherwise contribute only to the GAL4-TK-luc reporter. The homeodomain of PV.1 seems to be involved only in specific targeting but not to participate in repression because G4DBD-C-PV1 exerted the full activity without homeodomain.

When the C-terminus alone (G4DBD-C-PV1) is compared to the N-terminus alone (G4DBD-N-PV1) the former showed 40 times more the level of repression than that of the latter.

G4DBD-NH-PV1 or G4DBD-N-PV1 injection also downregulated the reporter gene and G4DBD-N-PV1 again showed a level of repression three times that of G4DBD-1/2N-PV1. This implies that a second repression module may exist in the upper 1/2N region. Xom, a member of another sub-family of Xvent, has been reported to have the repression domains at both the N- and C-terminii which also contains proline-rich residues [28]. The upper 1/2N region has a PHIPCAPQPLPPN K-YAKE sequence at its C-terminal end, which includes six prolines within 18 amino acids. In line with this, although the upper 1/2N region seemed to be dispensable

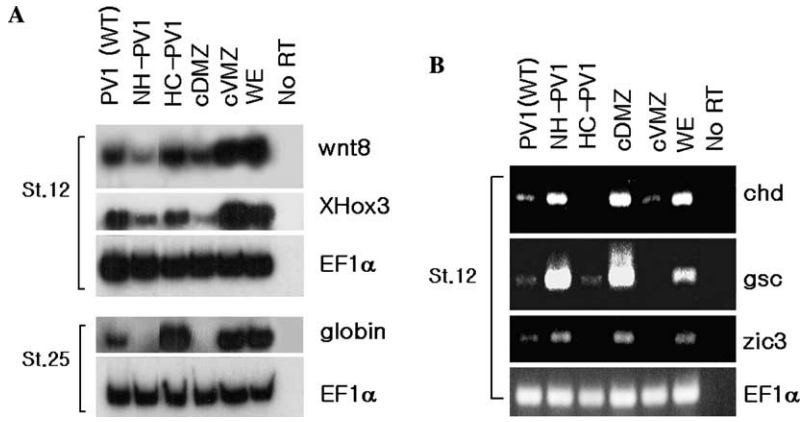

Fig. 4. HC-PV1 ventralizes dorsal mesoderm. Four-cell stage embryos were injected with *PV.1*, NH-PV1, and HC-PV1 mRNAs at their dorsal equatorial region. Dorsal marginal zone tissue was dissected at an early gastrula stage and cultured until the siblings reached stage 11 or stage 25 for RNA extraction. *PV.1* and HC-PV1 overexpression induces ventral marker genes (A) and suppresses dorsal specific early genes (B) while NH-PV1 fails to do this.

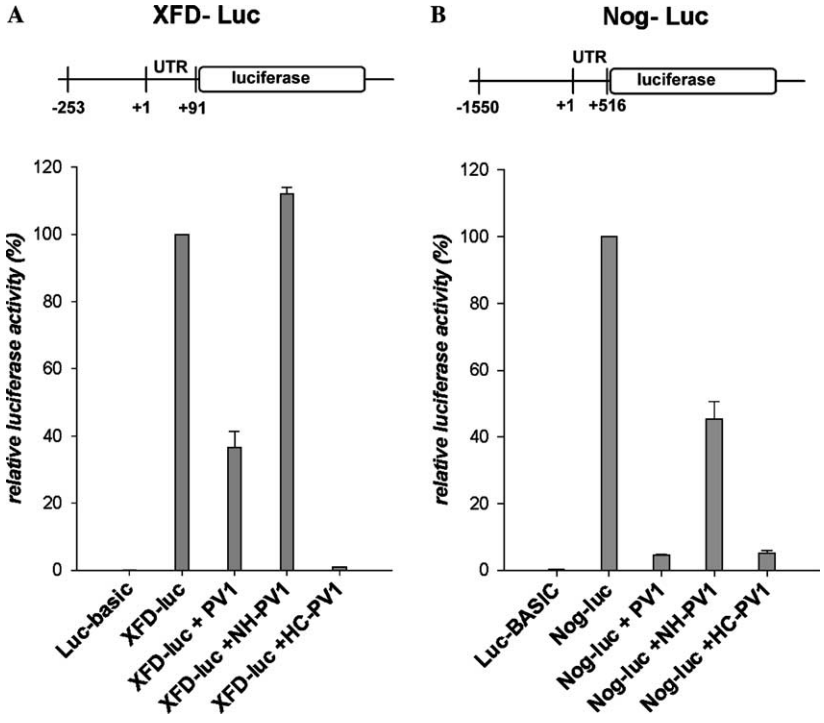

Fig. 5. Luciferase assay for *XFD-1′* promoter and Noggin promoter in response to deletion mutants of PV.1. mRNAs encoding NH-PV1, HC-PV1, and wild type *PV.1* (125 pg/blastomere, respectively) were co-injected with a reporter construct DNA (XFD-luc: 20 pg/blastomere, Nog-luc: 10 pg/blastomere; for details see Materials and methods) and reference plasmid DNA (pRL-TK; 10 pg/blastomere) at the dorsal equatorial region of four-cell stage embryos. Luciferase activity was measured at stage 12. Wild type *PV.1* reduced *XFD-1′* promoter (A) and *noggin* promoter (B) activity. HC-PV1 also reduced both promoter activities to the same level in *noggin* promoter assay (B) or to the lower level in XFD-luc promoter assay (A) as to wild type *PV.1*.

for ventralizing embryos, the mean DAI of 1/2NHC-PV1-injected embryos is a little higher than that with wild type PV.1 (Table 1).

These results indicate that the repressive activity of PV.1 is localized to the C-terminus and partly to the upper 1/2N region.

Characterization of the N- and C-terminal region of PV.1

To characterize the N- and C-terminal region of PV.1 we performed RT-PCR. Total RNA was isolated from the dorsal mesoderm of NH-PV1- or HC-PV1-injected embryos and assayed for expression of *Wnt8*, *Xhox3*,

globin, *Chordin*, *goosecoid*, and *Zic3*. The expression of *Xwnt-8*, which is primarily expressed in the prospective ventral-lateral mesoderm, *Xhox3*, ventroposterior marker gene, and *globin*, ventral-most blood marker, was induced in dorsal mesoderm tissue by an injection of HC-PV1 as by wild type, whereas NH-PV.1 did not induce ventral markers in the same tissue (Fig. 4A). Meanwhile, the expression of *Chordin*, one of the organizer secretory factors, *goosecoid*, the dorsal mesoderm-specific transcription factor, and *Zic3*, the pre-neural inducer, was significantly downregulated by a HC-PV1 injection in a similar fashion as a wild type *PV.1* injection. On the other hand, NH-PV1 injection did not change the expression of these dorsal genes (Fig. 4B). These results indicate that HC-PV.1, not NH-PV.1, mimics wild type PV.1 protein in ventralizing activity.

Since wild type PV.1 and HC-PV.1 caused ventralization of embryos and repression of organizer genes in dorsal mesoderm tissue, we then examined the repression of specific organizer genes using promoter/reporter constructs of the genes. *Xenopus* forkhead related gene (*XFD-1′*) is expressed within the organizer at the onset of zygotic expression and is induced directly by activin signaling. On the other hand, the expression of *XFD-1′* is repressed at the ventral/vegetal side and turned off indirectly by BMP-2/4 signaling. The expression mechanism of XFD-1′ has partially been demonstrated by the discovery that its promoter contains BIE (BMP-triggered inhibitory element) and ARE (activin responsive element) for the negative and positive regulation of its expression [39]. BMP-4 downstream mediator, Xvent-1, directly represses *XFD-1′* through binding to BIE [30].

PV.1 belongs to the Xvent homeobox multi-gene family and, more precisely, to the *Xvent-1* subfamily [11]. *PV.1* and *Xvent-1* show 84% nucleotide identity within their coding region. PV.1 ventralizes activin-induced dorsal mesoderm [12]. We, therefore, expected that PV.1 might also repress *XFD-1′* expression and examined the *XFD-1′* promoter responsiveness to two deletion mutants, NH-PV1 and HC-PV1 together with wild type PV.1.

XFD-1′ promoter reporter (XFD-luc) is highly activated when placed at the dorsal marginal zone (Fig. 5A). As expected, the PV.1 co-injection decreased the reporter activity. When HC-PV1 was co-injected into the dorsal side of the embryos, XFD-reporter activity was dramatically reduced, even more than that of wild type PV.1 co-injection. On the contrary, NH-PV1 did not reduce XFD-luc expression.

We then examined the repression activity of HC-PV.1 on another organizer gene, *noggin*. The 1556 base pair of 5′ flanking region of *noggin* has been reported to be active when placed in dorsal marginal zone. In animal cap explants, it is turned on only in response to activin or ArmdNXTcf-3, a constitutively active transducer of Wnt signaling pathway [31]. In our unpublished data, a PV.1 direct responsive element exists in this 1556 bp. Consistent with this, our previous RT-PCR data have shown that the expression of *noggin* was downregulated in whole embryos over-expressing PV.1 or HC-PV1 (data not shown). Co-injection with wild type PV.1 or HC-PV1 abolished the *noggin* promoter activity in the dorsal mesoderm tissue whereas NH-PV.1 showed half repression (Fig. 5B).

The function of serine-rich down 1/2N-domain is still unknown. It is unlikely that this region, by itself, contains an additional transacting repressor module because (i) this G4DBD-1/2N-PV1 exhibited three times less the level of repression than that of G4DBD-N-PV1 and almost no repression when compared to G4DBD-18a.a. (ii) The morphological appearance of HC-PV1-injected embryos differs from that of PV.1 or 1/2NHC-PV1-injected group in that they exhibit delayed or incomplete gastrulation (Fig. 2) i.e., down 1/2N region seemed to be required for restoring a specific effect of PV.1. (iii) In luciferase assay (Fig. 5), responsiveness of XFD-luc and Nog-luc to NH-PV1 was different. NH-PV1 retains at least half repression activity on the Noggin promoter but none on the *XFD-1′* promoter. Instead, the N-terminus seemed to rather activate this *XFD-1′* promoter in an antagonistic fashion to the repressive activity of C-terminus. A recent study has found that Xvent-2 and Xvent-2B physically interact with a cofactor complex, FLI/ERG [40]. Fli and erg are two members of the ETS gene family whose gene products act as transcriptional effectors in cell proliferation, differentiation, and oncogenic transformation. The serine-rich down 1/2N region, for this reason, may modulate their transcriptional activity by interacting with other ventralizing/dorsalizing co-factors thereby resulting in diverse spatiotemporal effect of PV.1.

Taken together, the present work has demonstrated that PV.1 represses organizer genes, as a transcriptional repressor which follows the active repression mechanism, and this repressive activity is localized to the C-terminus and partly to the upper 1/2N region.

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