Func tion al Anal y sis of the *Droso phi la* H731 Pro tein by the Yeast Two-hy brid Sys tem

Abstract *Drosophila* H731 (dH731) is the ho mo logue of mouse Pdcd4, which is im pli cat ed in apoptosis and tumorgenesis. In order to in ves ti gate the possible cellular functions of the dH731 protein, I at tempt ed to find out the in ter act ing pro teins of dH731 as well as to elucidate the functional re la tion ship be tween dH731 and its in ter act ing proteins. By yeast two-hybrid screening and database search, nine different dH731-interacting clones were isolated from 5×10 yeast clones. Five of them encode novel Droso phi la proteins, and two of the five have a C2H2 zinc finger motif which is a putative DNA binding domain. The other four proteins are dH731, dUBC9, rpL23a and eIF4a. Therefore, the data suggest that dH731 protein can bind to itself, forming homodimers or multimers. The mRNA expression patterns of the dH731 gene and its in ter act ing genes were examined by whole-mount in situ hy brid iza tion of the developing embryos. They were all localized in the central nervous system and partially overlapped in other tissues, including gonads. Taken together, it is conceivable that dUBC9 may help the dH731 protein enter the nucleus to interact with rpL23a and two other C2H2 zinc finger-containing proteins, and that the dH731 protein may play a role in regulation of transcription or cell cycle pro gres sion during the development of the nervous system and the reproductive organ. 6

1.Introduction

The *Drosophila* H731 protein (dH731) was iden ti fied through its in ter ac tion with Numb. Numb plays an im por tant role in determining cell fate dur ing neural de vel op ment (1)dH731 encodes 509 amino acid residues. The mouse Pdcd4 protein, which may in hib it tumor for ma tion (2)shows 41% identity to dH731, but the functions of dH731 have not been elucidated. Therefore, I am very in ter est ed in the functions of the dH731 protein dur ing embryonic development. The yeast two-hy brid sys tem (3) (Fig. 1) was used to search for the in ter act ing proteins of dH731, and nine dif fer ent proteins were found. The func tion al relationship between dH731 and its interacting pro teins was discussed.

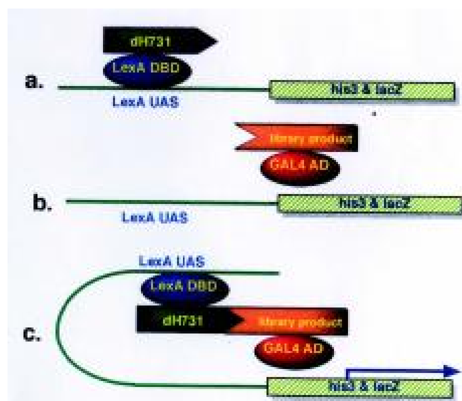
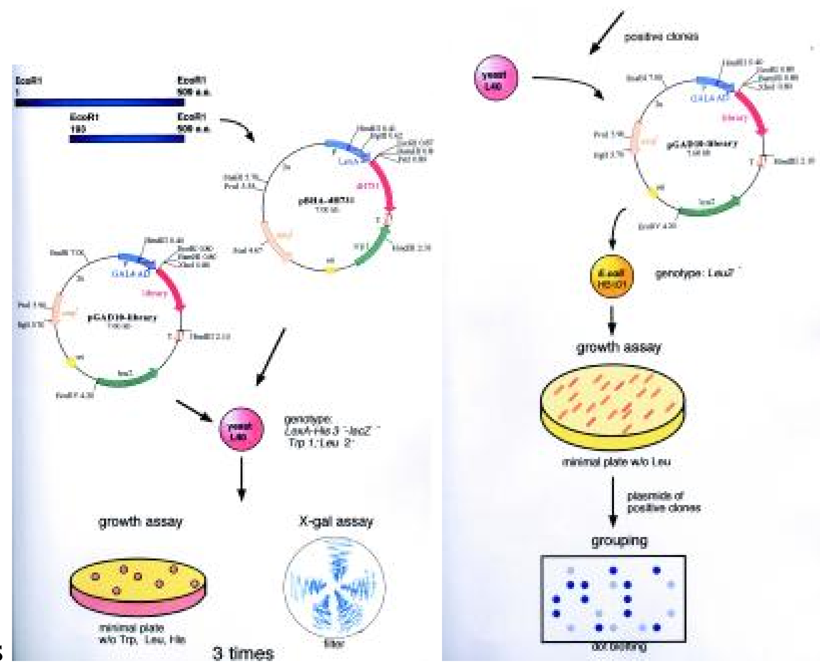


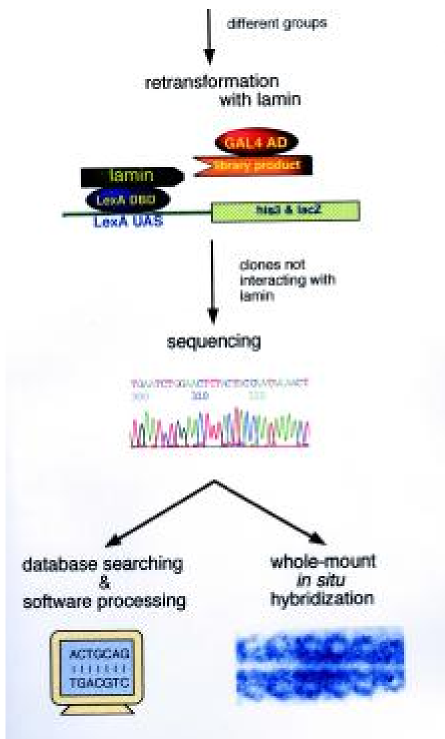
Fig. 1 Yeast two-hybrid system

2.Materials and Methods

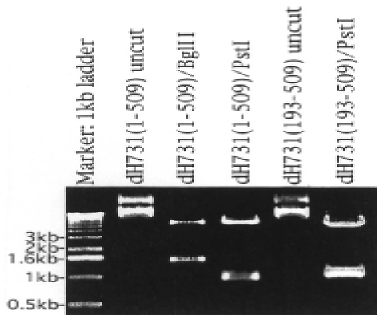
1. DNA cloning The DNA sequences of dH731, the full length and the C-terminus, were cloned into pBHA which contains se quenc es en cod ing the LexA DNA bind ing protein. The dH731 sequence was cloned down stream of LexA sequence (Fig.2).2. Yeast two-hybrid screening pBHA-dH731 and pGAD10-library were co-transformed into the L40 yeast with a heat shock pulse of 42℃ for 15 min. Positive transformants were con firmed repeatedly for three times of growth (for histidine synthesis) and X-gal assays (forβ-ga lac tosi dase activity). To pre vent more than one plasmid in a single yeast colony, pGAD10-library plasmids were ex tract ed from positive candidates, and trans formed into the HB101 *E. coli.* 3. Grouping and retransformation Since these positive candidates might con tain DNA sequence from the same gene, cross hy brid iza tion by dot blot ting was used to group the candidates. Non-radioactive DIG-labeled probes were generated and hybridization was per formed at 65℃. Representatives from each

group were retransformed into yeast L40 with pBHA-lamin to exclude non-specific interactors. Transformants that did not turn on the reporter genes with pBHA-lamin were the true positive. 4. Sequencing and database search The DNA sequences of dH731-interacting clones were subjected for sequencing. The resulting sequences were processed with softwares DNA Strider 1.2 and Gene Work 2.0, and were then compared to DNA sequence databases Berkeley Drosophila Genome Project, WWW BLAST at National Center for Biotechnology Information, Welcome to PubMed and ISREC ProfileScan Server. 5. Whole-mount embryo in situ hybridization The mRNA expression patterns of dH731 and its interacting genes were examined by whole-mount in situ hybridization of the developing embryos. The mRNA of these genes was hybridized by DIG-labeled probes which were recognized by anti-DIG-alkaline phosphatase (AP). These embryos were then subjected for color reactions to reveal the expression pattern.

Fig. 2 Schematic procedures 



3.Results 1. DNA cloning The DNA cloning results were ex am ined by aga r ose gel electrophoresis (Fig. 3). The three DNA sequence fragments of the dH731 gene were cloned to be in frame with LexA protein. These DNA in serts were also con firmed by sequencing.



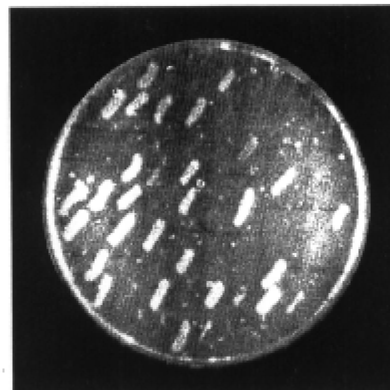
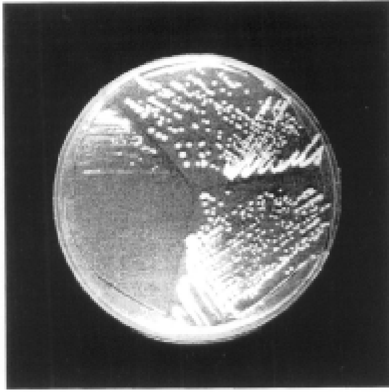
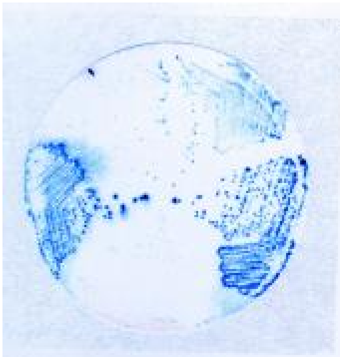
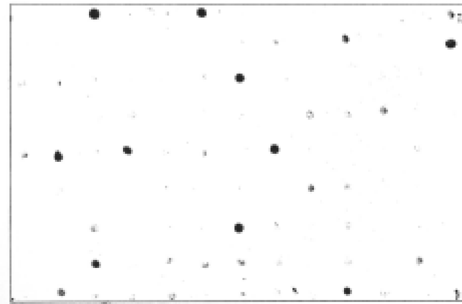


Fig. 3 Electrophoresis of dH731-pBHADNA fragments 2. Yeast two-hybrid screening Fig. 4 Growth assay of yeast for histi dine synthesis Fig. 5 X-gal assay for β-galactosi dase activity







6 Fig. 6 Selection of library plasmids (Leu2+) in *E. coli* HB101Fig. 7 Dot blotting to reveal identical DNA sequences pBHA-dH731 and pGAD10-library were co-trans formed into L40 yeast. The cDNA li brary from 0-3hr, 3-12hr em bry os and larva were used. Through three times of growth and X-gal assays, 133 pos i tive clones which could pro duce his ti dine and β-ga lac tosi dase were iso lat ed from 5×10 successfully transformed yeast clones. These can di dates were sub di vid ed into 26 dif fer ent groups

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by dot blotting. Finally, 9 in ter act ing pro teins of dH731 were found.
Table 1 Data of the yeast two-hy brid screen ing

Steps of the yeast two-hybrid screening	Total number of clones
Successful transformants	5,033,600
Interacting clones	245

First test	196
Second test	176
Third test	133

(Each test includes growth (Fig. 4) and X-gal assay (Fig. 5).)

Selection of library plasmids (*Leu2*$^+$) in *E. coli* HB101 (Fig. 6)	117

Dot blotting for clone grouping (Fig. 7)	26

Retransformation for selection true positive interactors	18

DNA sequencing	9

	dH731 interacting protein	Putative domains & Homologues	Open reading frame (a.a. residue)	Chromosomal localization	Proposed relationship with dH731
1	dH731	Bipartite nuclear localization signal (NLS) (a.a. 57-74)	509	12C	Forming homodimer
2	dUBC9	Ubiquitin-conjugating enzymes active site	158	21C6-7	Assisting dH73 in entering nucl
3	Ribosomal protein L23a	Bipartite NLS (a.a. 75-92, 96-113), Ala-, Lys-,Pro-rich	269	62B5-10	DNA binding
4	Initiation factor 4a	RNA helicase Vasa	403	67A2-B1	?
5	Novel #103	C_2H_2 zinc finger domain (a.a. 287-318)	321	92B-92C	DNA binding
6	Novel #148	C_2H_2 zinc finger domain (a.a. 144-166)	210	24C3-D2	DNA binding
7	Novel #143	Rat pyrophosphate decarboxylase (DNA sequence identity: 99%)	252	13E-13F	?
8	Novel #415	?	152	66A11-12	?
9	Novel #111	?	?	42D1-E2	?

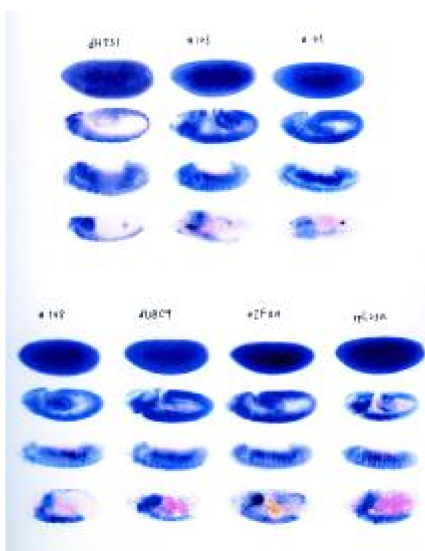

Fig. 8

tains a Bipartite NLS. It is considered that dH731 is able to enter the nu cle us because of its NLS, and that dUBC9 is in volved in this nu cle ar import process. In the nucleus, dH731 can interact with rpL23a and two novel proteins, #103 and #148, containing C2H2 zinc finger do main isolated in this screen ing (Table 2). The mRNA of dH731 is high ly expressed in em bry on ic neuroblasts, gonads and the central ner vous

4.Discussion By the way of the yeast two-hybrid screening, nine interacting proteins of dH731 were isolated. According to the expression patterns, cellular lo cal iza tion and functions of the dH731-in ter act ing proteins, the pos si ble func tions of dH731 were discussed. 1.dUBC9, one of the dH731 interacting proteins, interacts with several tran scrip tion factors, such as Tramtrack and Groucho, and is in volved in nuclear import. dUBC9 pro motes Dorsal, a tran scrip tion factor, entering nucleus (5). In dUBC9 mutant, Bicoid, a segment-re lat ed tran scrip tion factor fails to enter the nucleus and the seg ment of *Droso phi la* embryos is abnormal (6). Moreover, dH731 con

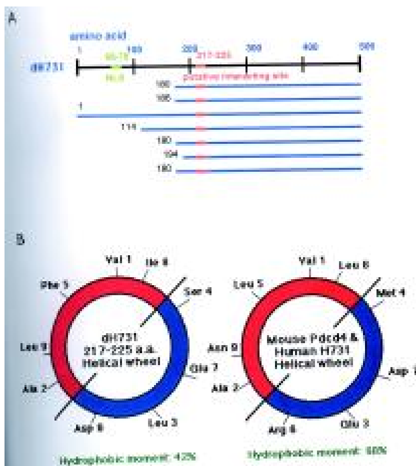

Fig. 9

sys tem (CNS) (Fig. 8). These cells are di vid ing and form ing organs in these stages of expression. The mRNA expression patterns of the dH731-in ter act ing genes are all lo cal ized in CNS, and par tial ly over lapped in other tissues, such as gonads. Taken together, it is con ceiv able that dH731 may enter nuclei with the assistance of dUBC9. Within the nucleus, dH731 interacts with the three DNA-binding proteins, rpL23a and two Zn finger pro teins (Fig. 10). These in ter ac tions may be im por tant for dH731 to play a role in reg u la tion of tran scrip tion or cell cycle pro gres sion during the neu ral de vel op ment and the re pro duc tive organ formation. Fig.10 The model for dH731 entering the nu cle us

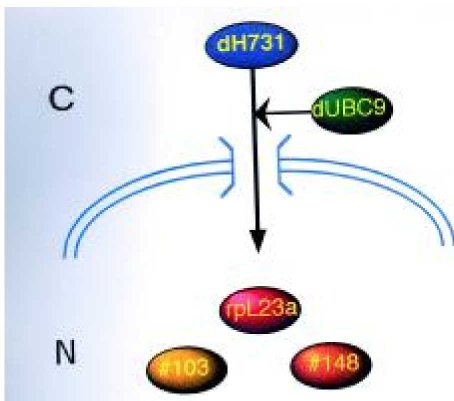

Fig.10

5.References 1. Cmarik J, Min H, Hegamyer G, Zhan S, Kulesz- Martin M, Yashinaga H, Matsuhashi S and Colburn N. (1999) Dif fer en tial ly expressed protein Pdcd4 inhibits tumor promotor-in duced neo plas tic transformation. *Proc. Natl Acad. Sci. USA*, 96, 14037-14042. 2. Fields S and Song O-K. (1989) A nov el ge net ic system to detect pro tein-pro tein interactions. *Nature*, 340, 245-246. 3.Bhaskar V, Valentine SA and Courey AJ. (2000) A functional in ter ac tion between Dorsal and com po nents of the Smt3 conjugating machinery. *J. Biol.* Chem. 275, 4033-4040.