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Expression of Cell Proliferation-Related PCNA and E2F Genes in *Drosophila* Gut and Inhibitory Effect of Nitric Oxide

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To understand the late gut development and differentiation, identification and characterization of target genes of homeotic genes involved in gut development are required. We have previously reported that homeodomain proteins can regulate expression of the cell proliferation-related genes. We investigated here the expression of the *Drosophila* proliferating cell nuclear antigen(PCNA) and E2F(dE2F) genes in larval and adult guts using transgenic flies bearing *lacZ* reporter genes. Both PCNA and dE2F genes were expressed strongly in whole regions of the larval and adult guts including the esophagus, proventriculus, midgut and hindgut, showing higher expression in foregut and hindgut imaginal rings of larva. Nitric Oxide(NO) has been known to be involved in cell proliferation and tumor growth and also to have an antiproliferative activity. Therefore, we also investigated effects of NO on the expression of PCNA and dE2F genes in gut through analyses of *lacZ* reporter expression level in the SNP (NO donor)-treated larval guts. Expressions of both PCNA and dE2F were greatly declined by SNP. The inhibitory effect of NO was shown in whole regions of the gut, especially in hindgut, while the internal region of proventriculus, esophagus, foregut imaginal ring and hindgut imaginal ring was resistant. Our results suggest that this inhibitory effect may be related with the antiproliferative activity of NO.

The alimentary canal of *Drosophila* is composed of three major parts; the foregut, midgut and hindgut. The foregut is subdivided largely into the pharynx, esophagus and inner part of the proventriculus. The midgut includes the outer wall of the proventriculus and a major central portion. The hindgut is composed of the posterior part of large intestine and the rectum (only in adult). The foregut and hindgut epithelia arises from an ectodermal primordium and the midgut epithelium originates from the endoderm. All the gut epithelia derive from invagination of two separate domains, the anterior and posterior termini of the blastoderm. The anterior invagination results in the formation of the foregut and anterior midgut. The posterior invagination forms hindgut, malpighian tubule and posterior midgut (Michael bate et al., 1993). The anterior and posterior terminal domains of the early blastoderm are under the control of sequential steps of gene activation including *tailless, huckebein, serpent* and *brachyenteron* (Murakami et al., 1998). The first step is establishment of graded activities of maternal gene products such as transmembrane receptor tyrosine kinase Torso, that provides positional information on the anterior and posterior terminal domains (Greenwood and Struhl, 1997). The second step is interpretation of the positional information by the zygotic gap genes *tailless* and *huckebein,* which largely subdivide the terminal domains. The third step is specification of each subdomain as a primordium of gut parts by genes that work downstream from the gap genes: *serpent* for the anterior and posterior endoderms (Rehorn et al., 1996) and *brachyenteron* for the ectoderms (Kispert et al., 1994). In the specification of the foregut and hindgut from the ectoderm, many segmentation genes such as *wingless, hedgehog* and *decapentaplegic* (Skaer, 1993; Hoch and Pankratz, 1996; Singer et al. 1996 for review) and homeobox genes, such as *engrailed* and *caudal* (Linda et al., 1998) are expressed. However activities of the genes involved in the subdivision of the gut and in the coordination of its differentiation remain unknown. To understand the late gut development and differentiation, identification and characterization of target

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genes of homeotic genes involved are required. Recently, abnormal expression pattern and function of a homeobox gene in the gut has been known to provoke colon cancer, in which cells escape proliferation control and display abnormal differentiation (Vider et al., 2000). It has been reported that homeodomain proteins can affect promoter activities of the cell proliferation-related genes in cultured cells and in living organisms (Yamaguchi et al., 1995; Ryu et al., 1997; Um et al., 1998). Therefore, to understand the late gut development and differentiation, we were first interested in expression patterns of the cell proliferation-related genes, proliferating cell nuclear antigen(PCNA) and E2F(dE2F), in *Drosophila*. It has been reported that PCNA gene is expressed in the salivary gland, brain lobe, imaginal disc and ovary of larvae and adult (Yamaguchi et al., 1995). In the case of dE2F gene, expression in the central nervous system, neuroblast of embryo (Duronio et al., 1995) and eye imaginal disc of larvae (Brook et al., 1996) have been reported. Until now, however, there has been no information about expression of these genes in *Drosophila* gut.

Recently, relatively high nitric oxide(NO) production rates in human colon carcinoma tissues have been reported (Ambs et al., 1998; Kojima et al., 1999). NO is a free radical gas, known to be involved in cell proliferation and tumor growth (Yamaguchi et al., 2000) and also reported to be an efficient antiproliferative agent (Lepoivre et al., 1990). We report expression patterns of the cell proliferation-related genes PCNA and E2F in *Drosophila* larval and adult guts and inhibitory effect of NO on their expression in the gut using transgenic files bearing *lac*Z-reporter genes.

Materials and Methods

Fly strains

To examine PCNA gene expression, we used p5'-168DPCNA *lacZ*W8HS transgenic line (Yamaguchi et al., 1991) carrying PCNA promoter gene fragment spanning from -168 to +137 fused with *lacZ* in P-element vector. The promoter region (-168 to +24 placed upstream of the transcription initiation site) is practically sufficient for expression of the PCNA gene (Yamaguchi et al., 1995). To examine dE2F gene expression, we used $dE2F^{rm729}$line in which P element-*lacZ* is inserted 48 bp upstream of the initiation site of dE2F gene, with the P element-*lacZ* in the same orientation as dE2F gene (Duronio et al., 1995), named E2F-*lacZ*. *LacZ* expression was therefore directed by the dE2F gene promoter and its pattern was indistinguishable from the expression of dE2F (Brook et al., 1996). pMT-*lacZ* transgenic line (Kim et al., 1998) carrying metallothionein(MT) promoter region 378 bp fragment (-320 - +58 with respect to the transcription initiation site) fused with *lacZ* was used as control in SNP-treatments. The *white* and ry^{506} flies were used as wild type. Flies were maintained in culture bottles containing standard cornmeal agar medium (8 g agar powder, 100 g sugar, 70 g corn-meal, 15 g ebiose, 15 g dried yeast powder, 3 ml bokinin, 4 ml propionic acid and 1 L water).

X-Gal staining

β-Galactosidase activities in larval and adult guts were visualized as described elsewhere. The third instar larvae and adults were washed with Ringer's solution (182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.2)) and dissected with microforceps. The dissected larvae and adults were incubated in fixation buffer (1% glutaraldehyde in 1×PBS) for 15 min on ice. The samples were then washed three times every 10 min with 1×PBS (phospate-buffed saline; 130 mM NaCl, 7 mM $Na_2HPO_42H_2O$, and 3 mM $NaH_2PO_42H_2O$) and incubated with 0.2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in dark at 37℃ for appropriate time periods. The larvae were incubated for 5 h to 16 h and adult for 2 h to 5 h. To correct for endogenous *lacZ* expression, wild type flies (the *w* and ry^{506} strain) were processed at the same time. The guts were dissected out, immersed in Crystal mountant, mounted on slides and photographed with Karl Zeiss TK-C1380 microscope.

Quantitative measurement of β-galactosidase activity

Quantitative measurements of β-galactosidase activity in extracts were carried out as described previously (Fridell et al., 1992). The larval and adult guts were dissected out with microforcep and homogenized in 100 μl assay buffer (50 mM potassium phosphate, and 1 mM $MgCl_2$ (pH 7.5)). The homogenates were centrifuged at 13,000 rpm at 4℃ for 10 min. For the assay, the supernatant was mixed with 200 μl of assay buffer contaning 1 mM CPRG substrate (Boehringer Mannheim) and incubated at 37℃ in dark. Substrate conversion was measured at 574 nm using a spectrophotometer 0.5, 1, 1.5 h after the addition of the extract and the rate of color development was linear. The β-galatosidase activity was defined as absorbance unit/h/mg of protein. To correct for endogenous β-galactosidase activity, extracts from the host strain were included in each experiment, and background reading was subtracted from reading obtained from each transformant line.

SNP (NO donor) treatment

Guts of the third instar larvae were dissected out in M3 media on ice and treated with 30 μM, 50 μM and 100 μM SNP(sodium nitroprusside) for 6 h or 3 h at 25℃ in the dark. X-gal staining and CPRG assays were carried out immediately afterwards.

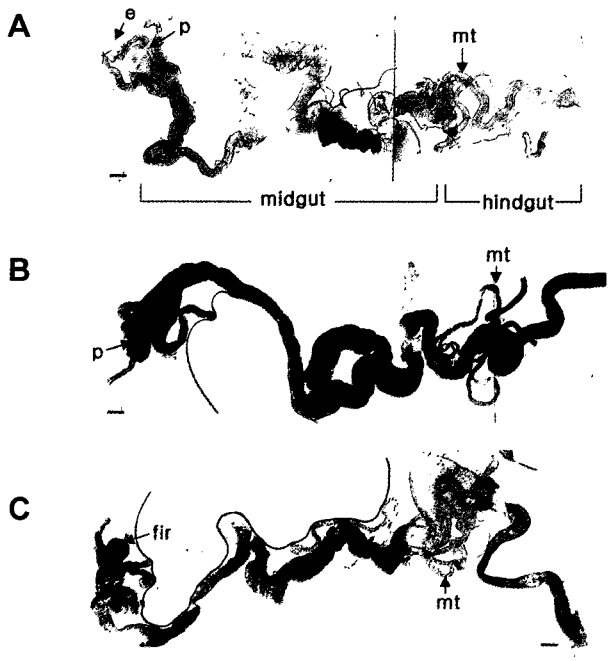

Fig. 1. The expression patterns of PCNA and E2F genes in larval gut. Third larval guts having *lacZ* fusion gene were dissected and stained in 0.2% X-gal solution for 5 h at 37℃. A, Endogenous *lacZ* expression in larval gut of nontransgenic *w*. B, PCNA-*lacZ* expression in larval gut carrying p5'-168DPCNA*lacZ*W8HS. C, E2F-*lacZ* expression in $dE2F^{rm729}$ larval gut. e, esophagus; fir, foregut imaginal ring; mt, malpighian tubule; p, proventriculus. Scale bars=50 μm.

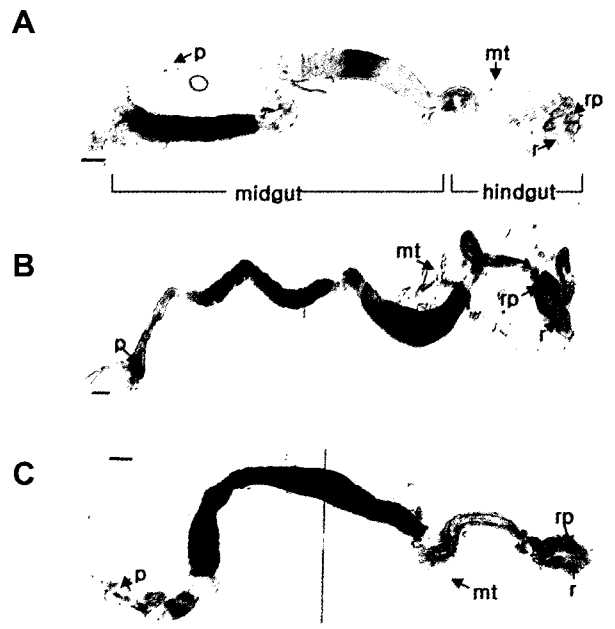

Fig. 2. The expression patterns of PCNA and E2F genes in adult gut. The adult guts having *lacZ* fusion gene were dissected and stained in 0.2% X-gal solution for 5 h at 37℃. A, Endogenous *lacZ* gene expression in adult gut of nontransgenic *w*. B, The PCNA-*lacZ* gene expression in adult gut carrying p5'-168DPCNA*lacZ*W8HS. C, E2F-*lacZ* gene expression in $dE2F^{rm729}$ adult gut. mt, malpighian tubule; p, proventriculus; r, rectum; rp, rectal papillae. Scale bars=50 μm.

Results

Expression patterns of PCNA-lacZ and E2F-lacZ genes in third larval gut

Expression patterns of PCNA and dE2F genes in the larval gut were examined by x-gal staining of larval guts of transgenic flies carrying p5'-168DPCNA*lacZ*W8HS and of $dE2F^{rm729}$flies (Fig. 1). Endogenous β-galactosidase expression in the wild type was shown in partial regions of the midgut (Fig. 1A). The PCNA-*lacZ* gene was expressed intensively in all regions of the larval gut including the esophagus, proventriculus, midgut, hindgut and malipighian tubule, with higher expression in the imaginal rings of the foregut and hindgut (Fig. 1B). E2F-*lacZ* gene was expressed in the esophagus, proventriculus and anterior, central midgut and hindgut (Fig. 1C) and also intensively in the imaginal rings. Expression pattern of E2F-*lacZ* showed staining of nucleus in all gut cells.

Expression patterns of PCNA-lacZ and E2F-lacZ genes in the adult gut

Expression patterns of PCNA and E2F genes in the adult gut were also examined. Endogenous β-galactosidase expression in the wild type was shown weakly in the central region and strongly in the anterior part of the midgut (Fig. 2A). PCNA-*lacZ* gene was expressed in the internal parts of the proventriculus, midgut, hindgut and specifically rectal papillae of rectum (Fig. 2B). The proventriculus organ corresponds to mammalian stomach and is divided into internal and external regions. The internal region is occupied with the end of esophagus which corresponds to the foregut originating from the ectoderm. The external region corresponds to the midgut region originating from the endoderm. The rectum is specialized from the posterior hindgut.

E2F-*lacZ* gene was expressed in the inner part of the proventriculus, whole midgut, hindgut and the rectal papillae of rectum (Fig. 2C). Its expression in adult gut was broad, compared to the nucleus staining in the larval gut. There was no differences between female and male.

Inhibitory effect of NO on expression of the cell proliferation-related genes PCNA and E2F in third larval gut

NO is a multifunctional second messenger, which has been known to be involved in cell proliferation and tumor growth and also to have an antiproliferative activity. Here we investigated effect of NO on the expression of the cell proliferation-related genes PCNA and E2F which are expressed only in proliferating cells. We first examined expression levels of PCNA-*lacZ* and E2F-*lacZ* in the SNP (NO donor)-treated guts of third larvae by quantitative measurement methods. Expression of both PCNA-*lacZ* and E2F-*lacZ* genes

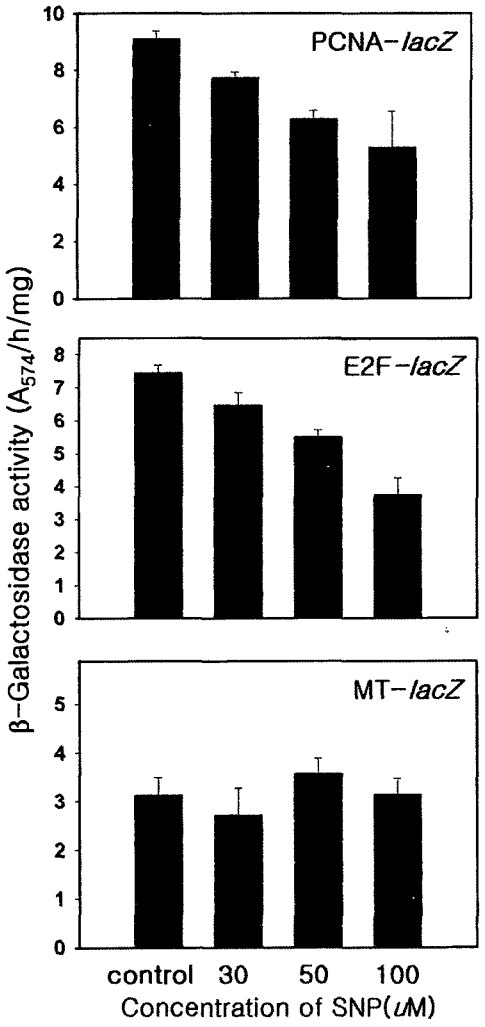

Fig. 3. Effects of nitric oxide on the expression of PCNA and E2F genes in larval gut. The expression level of PCNA-*lacZ*, E2F-*lacZ* or MT-*lacZ* gene was quantified with β-galactosidase activity of transgenic flies bearing the PCNA-*lacZ*, E2F-*lacZ* or MT-*lacZ* fusion genes. The guts of PCNA-*lacZ*, E2F-*lacZ* or MT-*lacZ* 3rd larvae were dissected, treated with SNP 30 μM, 50 μM or 100 μM in dark condition for 6 h at 25 ℃ and examined for β-galactosidase activity using 1 mM CPRG substrates. Substrates conversion was measured at 574 nm using a spectrophotometer at 0.5, 1 and 1.5 h after addition of extract, and the rate of color development was linear. The activity in the extracts was defined as absorbance units/h/mg of protein. Averaged values obtained from seven independent experiments. Value are given to β-galactosidase activity.

were declined by SNP (Fig. 3). Their expression were reduced to 50% of control by SNP 100 μM. To determine whether this decline was due to SNP toxicity in tissue, we examined expression of MT-*lacZ* of transgenic gut carrying MT promoter region fused with *lacZ* gene as control. Even at 100 μM SNP, the expression of MT-*lacZ* gene in SNP-treated gut was not decreased as compared with control. This suggested that the decreased expressions of PCNA and E2F genes by SNP were not due to SNP toxicity but were due to an inhibitory effect of NO on their expression. Patterns of the inhibition were examined by X-gal staining after SNP 100 μM treatment for 3 h. PCNA-*lacZ* gene expression was decreased in the whole gut (Fig. 4). Especially in the hindgut, the PCNA-*lacZ* gene expression was extremely decreased. In the proventriculus, it was shown weakly in the internal region. The foregut imaginal ring seemed to be resistant to the exogenous NO. The hindgut imaginal ring and malpighian tubules were also resistant (Fig. 4F). E2F-*lacZ* expression was also greatly decreased in almost all regions of the gut except for the internal region of the proventriculus, esophagus, foregut imaginal ring and hindgut imaginal ring (Fig. 5D, E, and F). Similar to PCNA-*lacZ*, E2F-*lacZ* expression in the hindgut was extremely reduced. This reduced expression pattern by SNP was similar to that of PCNA gene in its region and degree of decline.

Discussion

In the specification of the foregut and hindgut from ecdoderm, many segmentation genes such as *wingless, headgehog* and *decapentaplegic* and homeobox genes such as *engrailed* and *caudal* express during development (Skaer, 1993; Hoch and Pankratz, 1996; Singer et al., 1996 for review). To understand the late gut development and differentiation, identification and characterization of target genes of these genes are required. We previously revealed that *engrailed* homeodomain protein could affect promoter activity of *Drosophila* PCNA gene in cultured cells (Um et al., 1998). The proliferating cell nuclear antigen (PCNA) is an auxiliary protein for DNA polymerase δ (Bravo et al., 1987) and important for cellular DNA synthesis and cell progression (Jaskulski et al., 1988). Expression of PCNA gene in salivary gland, brain lobe, imaginal disc and ovary of larvae and adult have been reported (Yamaguchi et al., 1995). E2F transcription factors have been identified as key downstream factors of the Retinoblastoma (Rb) family members, playing essential roles in regulation of the correct timing of activation of S phase genes (Nevins et al., 1992). In mammals, at least six E2F family members (E2F1, E2F2, E2F3, E2F4, E2F5, and E2F6) and two dimmer partners (DP1 and DP2) have been characterized (Bandara et al., 1993; Helin et al., 1993; Hijmans et al., 1995; Vario et al., 1995; Trimarchi et al., 1998). In *Drosophila,* dE2F, dE2F2 and DP have been characterized (Ohtani et al., 1994) and found to play a role in development and cell proliferation by DNA-binding (Ohtani et al., 1994). It was reported that dE2F regulates PCNA promoter activity during development (Yamaguchi et al., 1995) and embryos lacking both maternal and zygotic RBF show constitutive expression of PCNA gene (Du et al., 1999). Expression of dE2F gene in the central nervous system, neuroblast of embryo (Duronio et al., 1995) and eye imaginal disc of larvae have been reported (Brook et al., 1996). The present results show that PCNA and E2F genes are expressed in are regions of larval and adult guts (Fig. 1 and Fig. 2). Especially

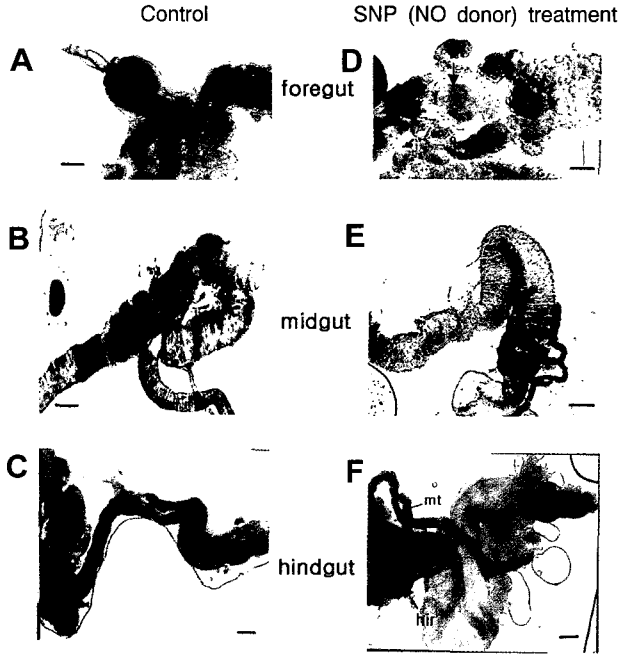

Fig. 4. Effects of exogenous nitric oxide on the expression pattern of PCNA-*lacZ* gene in larval gut. Effect of SNP on the expression pattern of PCNA-*lacZ* gene was analyzed by X-gal staining. The guts of p5'-168DPCNA*lacZ*W8HS 3rd larvae were dissected, treated with SNP 100 μM in dark condition for 3 h at 25℃ and stained in 0.2% X-gal solution for 5 h at 37℃. A-C, Control. D-F, SNP-treated gut. Strong inhibitory effect of NO on the PCNA-*lacZ* expression was detected in whole gut, especially hindgut. hir, hindgut imaginal ring; mt, malpighian tubule; p, proventriculus. Scale bars=100 μm.

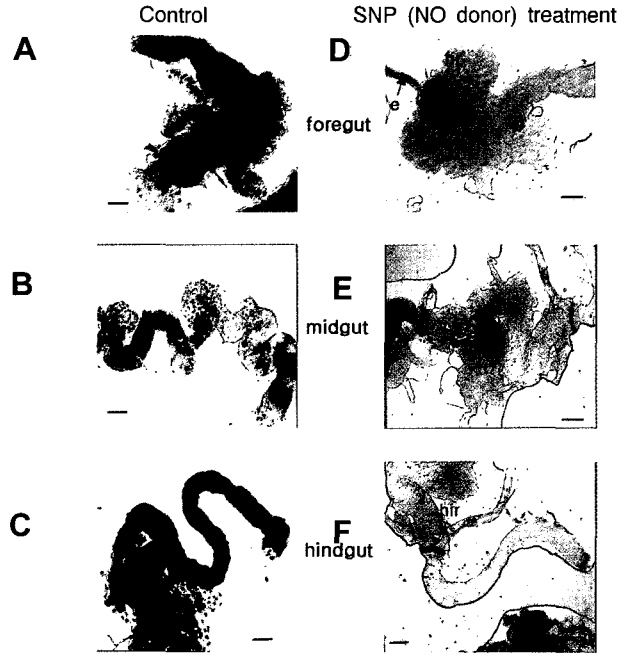

Fig. 5. Effects of exogenous nitric oxide on the expression pattern of E2F-*lacZ* gene in larval gut. Effect of SNP on the expression pattern of E2F-*lacZ* gene was analyzed by X-gal staining. The guts of *dE2F*^{rm729} 3rd larvae were dissected, treated with SNP 100 μM in dark condition for 3 h at 25℃ and stained in 0.2% X-gal solution for 5 h at 37℃. A-C, Control. D-F, SNP-treated gut. Strong inhibitory effect of NO on the E2F-*lacZ* expression was detected in whole gut, except imaginal rings. e, esophagus; fir, foregut imaginal ring; hir, hindgut imaginal ring. Scale bars=100 μm.

higher expression of these genes were detected in the imaginal rings of larval gut which are the actively proliferating region.

Nitric oxide (NO), a multifunctional second messenger, activates sGC (soluble guanylyl cyclase) which in turn stimulates synthesis of cyclic GMP and downstream cascade leading to multifunction (Grag and Hassid, 1989). Extensive expression of NOS in digestive tract (Vanderwinden et al., 1994) and diverse pattern of NOS gene expression and nitric oxide generation in human colon cancer cell lines (Jenkins et al., 1994) were reported. NO can act in cells as an antiproliferative agent via suppression of DNA synthesis (Grag and Hassid 1989; Lepoivre et al., 1990; Kwon et al., 1991). It was elucidated that inhibition of NOS causes hypertrophy of organ in adult flies, whereas ectopic expression of NOS in larvae has the opposite effect (Kuzin et al., 1996). However, the exact mechanism of the antiproliferative activity of NO in developing organism remains unknown. Our results show that NO has inhibitory effects on the expression of PCNA and E2F genes in the gut. The hindgut was especially sensitive to the inhibitory effect of NO, while the proliferation-active imaginal rings of gut was resistant. We suggest that the antiproliferative activity of NO may be due to inhibition of expression of the cell proliferation-related genes PCNA and E2F.

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