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Presence of a Regulatory Element within the First Intron of the Human Platelet-derived Growth Factor-A Chain Gene

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We detected a suppressive element in the first intron of the human platelet-derived growth factor A chain (PDGF-A) gene. Two or more proteins, at least 110-kd and 90-kd proteins, were bound over a wide region of this fragment, and the fragment suppressed the expression of the PDGF-A chain via these proteins *in vivo*. Since the fragment also had suppressor activity on the promoter of the PDGF-B chain, it may be involved in a suppressive mechanism of gene expression common to PDGF-A and -B chains. Four tandem repeats of CCCCAT(CCCC) and three direct repeats of GGGGAG were observed in this region. The expression of the PDGF-A chain is considered to be regulated by a mechanism involving not only the 5′ upstream region but also introns.

Key words: PDGF-A — Gene regulation — Intron — Suppressor

Platelet-derived growth factor (PDGF) is a heterodimeric glycoprotein consisting of A and B chains, and is one of the major mitogens of cells derived from mesodermal tissues.[1] The PDGF-B chain is a normal cellular homologue of v-*sis*, which is the oncogene of simian sarcoma virus,[2,3] and the PDGF-A chain is expressed in many tumor cells.[4] Abnormalities in the expression of these genes are considered to be closely related to the process of carcinogenesis. The regulatory mechanism of the expression of the PDGF-A chain gene is very complicated. Although the regulatory site in its 5′ region was identified,[5] the mechanism of regulation remains to be clarified. Recently, the presence of positive and negative regulatory elements has been demonstrated in introns of various genes.[6–14] We report here an element with strong suppressor activity detected in the first intron of the PDGF-A chain gene.

MATERIALS AND METHODS

Construction of plasmids Plasmids shown in Fig. 1 were prepared by fusing PSVOCAT containing the structural gene of chloramphenicol acetyl transferase (CAT) and fragments of the PDGF-A chain gene digested with various restriction enzymes. CAT, Xh-S included the 5′ region (−254 to +387) of the PDGF-A chain,[5] and 5′-PDGF-B, CAT included the 5′ region of the PDGF-B chain (−1374 to +90).[15] PSV2CAT included the promoter and enhancer sequences of SV40, and PA10CAT2 included the promoter sequence of SV40.

DNA transfection and CAT assay About 24 h before transfection, RD cells (a human embryonal rhabdomyosarcoma cell line) were seeded at 5.5×10^5 cells per 100-mm Petri dish. Twenty micrograms of each plasmid was transfected by the $CaPO_4$ method.[16] After 3 h, the cells were treated with 15% (v/v) glycerol in 20 m*M* Hepes buffer for 3 min, washed, and incubated for 48 h with 10% fetal calf serum (FCS). The cells were harvested, and 100 μg of lysate was assayed for CAT activity.[17]

Gel retardation assay Nuclear protein (NP) was extracted from RD cells incubated with 10% FCS by the method of Shapiro *et al.*[18] Suspended cells after hypotonic treatment were destroyed using a Dounce homogenizer. The nucleus was obtained by centrifugation, and nuclear protein was extracted with ammonium sulfate. For gel retardation assay, a ^{32}P-labeled DNA fragment (25,000 cpm), 5 μg of nuclear protein, and 5 μg of poly (dI-dC) were mixed in 25 μl of binding buffer (10 m*M* Hepes, pH 7.9; 60 m*M* KCl; 7.5 m*M* $MgCl_2$; 0.1 m*M* EDTA; 1 m*M* DTT; and 10% glycerol), incubated at room temperature for 30 min, and subjected to electrophoresis (10 V/cm) with 5% polyacrylamide gel (PAGE).

Photo-affinity labeling Photo-affinity labeling was performed by the method of Wu *et al.*[19] Plasmids containing the fragment of the intron were digested with restriction enzymes at the 5′ (for labeling of the lower strand) or 3′ (for labeling of the upper strand) site. After alkaline denaturing, fragments were labeled by annealing with primers and filling the strand with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP, dATP, dGTP and 5′-bromo-2′-deoxy uridine triphosphate (BrdU) according to standard procedures. Plasmids were digested with restriction enzymes at another site of the intron fragments and fragments were purified after elec-

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trophoresis for the binding reaction. The binding reaction was done by the same procedure as in the case of gel retardation assay except that the ^{32}P-BrdU fragments were used. The open microfuge tubes were then placed under (5 cm sample-to-lamp separation) an inverted 302-nm ultraviolet transilluminator and irradiated at 4°C for 40 min, with agitation every 10 min. The samples were digested for 30 min at 37°C with 10 m*M* $CaCl_2$, 10 m*M* $MgCl_2$ and 1 μg of DNase I. The reaction was terminated with 15 m*M* EDTA, 10% SDS-PAGE and autoradiography was done according to standard procedures.

RESULTS

Effects of the intron on the promoter region of the PDGF-A chain The fragment P-A and N-N in the first intron markedly suppressed the CAT, Xh-S activity in RD cells (Fig. 1). This suggests that elements that potently suppress the expression of the PDGF-A chain gene are contained in the N-N fragment. This suppressor activity was independent of the orientation of the N-N fragment. However, when the N-N fragment was divided into 4 small fragments, its suppressor activity disappeared (Fig. 2). Therefore, a wide region and/or the regions around *Alu* I sites of the N-N fragment are considered to be needed for the suppressor activity to appear.

Presence of nuclear proteins binding to fragments in the intron By gel retardation assay, specific bands, which were inhibited by the competitor, were observed in the N-N fragment (Fig. 3). Similarly to CAT assay, gel retardation assay was performed after digesting the N-N fragment into 4 segments, and specific bands inhibited by the competitor were observed in each fragment (Fig. 4). Therefore, nuclear proteins bind to the wide region of the N-N fragment. Photo-affinity labeling was performed to

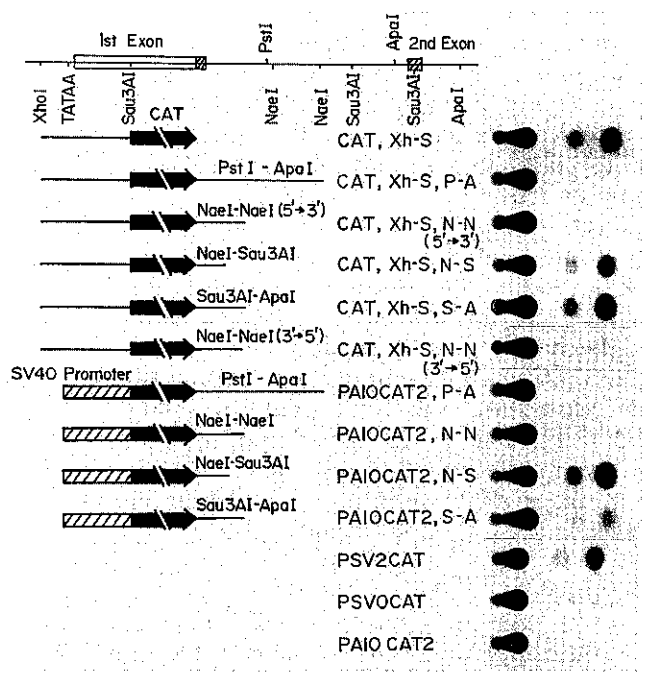

Fig. 1. Effects of the intron on the PDGF-A promoter (CAT, Xh-S) and SV40 promoter (PA10CAT2). Various plasmids were transfected into RD cells and CAT assay was carried out after incubation for 48 h in 10% FCS.

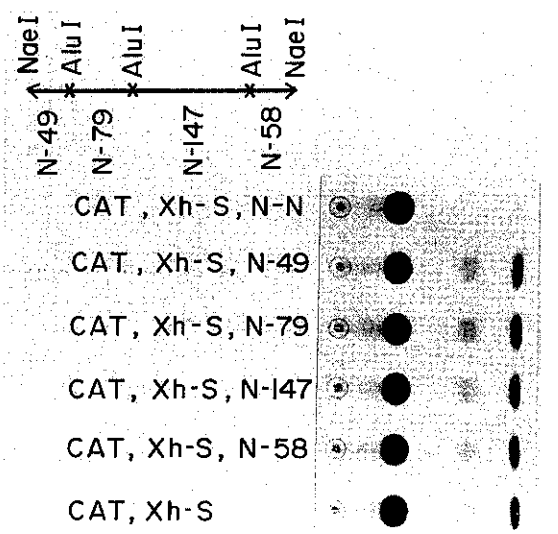

Fig. 2. The suppressor activity disappeared when N-N fragments were further fragmented to N-49, N-79, N-147, and N-58.

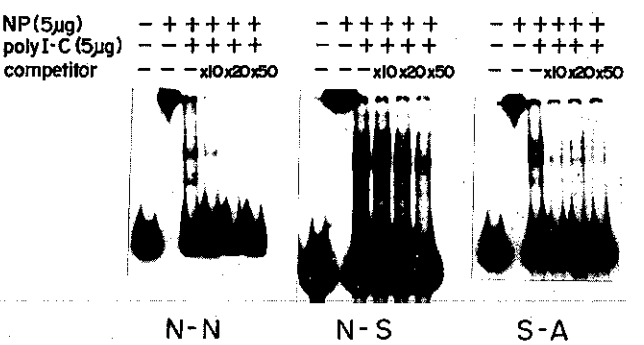

Fig. 3. Gel retardation assays. N-N, N-S, and S-A fragments all showed specific bands inhibited by the competitor.

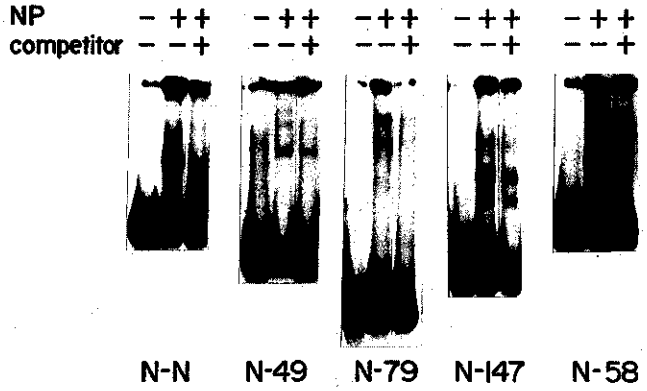

Fig. 4. Gel retardation assays were performed with N-49, N-79, N-147, and N-58 fragments. Specific bands inhibited by the competitor were observed in all these fragments.

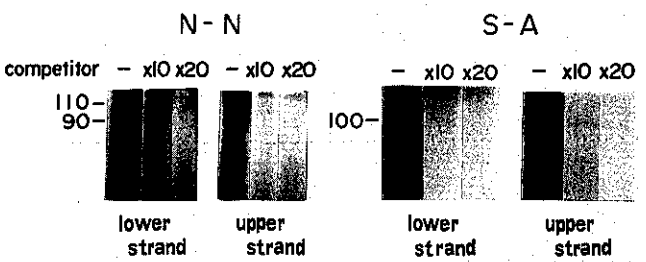

Fig. 5. Photo-affinity labeling. The 110-kd and 90-kd nuclear proteins were bound directly to the N-N fragment. A 100-kd protein was labeled in the S-A fragment.

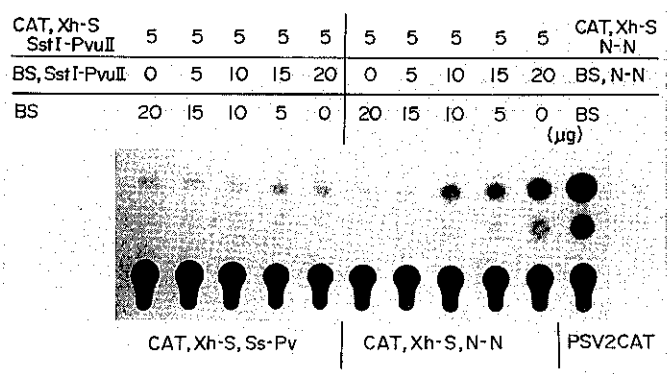

Fig. 6. Intracellular competition assay. Co-transfection was carried out using a total of 25 μg of plasmid by mixing 5 μg of CAT, Xh-S, N-N with the BS, N-N plasmid containing only the N-N fragment and the Bluescript plasmid (BS) without the fragment. The CAT activity increased in proportion to the amount of BS, N-N. The *SstI-PvuII* fragment (-618 to -281)[5] was used as a negative control.

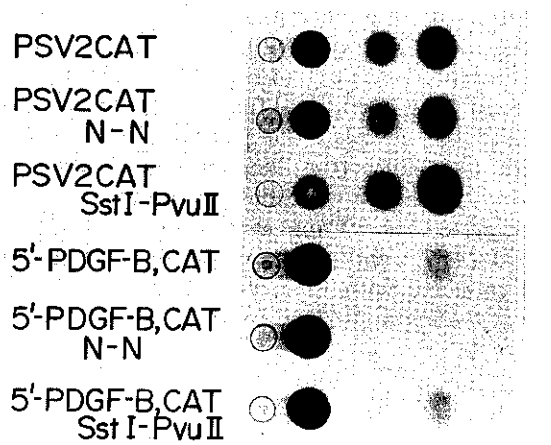

Fig. 7. Effects of PDGF-A chain introns on heterogenic promoters and enhancers. The N-N fragment had little effect on the SV40 promoter and enhancer (PSV2CAT) but suppressed the PDGF-B chain promoter and enhancer (5′-PDGF-B, CAT).

clarify whether the binding between these proteins and the N-N fragment was direct or not. As shown in Fig. 5, 110-kd and 90-kd nuclear proteins were bound directly.

Relations between proteins bound and the suppressor activity Whether these proteins bound to the N-N fragment are related to the suppressor activity *in vivo* was examined by intracellular competition assays (Fig. 6). The suppressor activity of CAT, Xh-S, N-N decreased as the competitor was increased, suggesting that the proteins bound to the N-N fragment also induce the suppressor activity of the fragment *in vivo*.

Effects of the PDGF-A chain intron on heterogenic promoters and enhancers The N-N fragment had little effect on the SV40 promoter and enhancer but showed suppressor activities on the PDGF-B chain promoter and enhancer (Fig. 7), similar to those in the case of the PDGF-A chain. Interestingly, N-S and S-A fragment showed a positive activity towards the SV40 promoter in RD cells (Fig. 1). In these fragments, gel retardation assays revealed specific bands inhibited by the competitor, and a 100-kd protein was labeled by photo-affinity labeling using the S-A fragment (Fig. 5).

DISCUSSION

This study showed that the N-N fragment of the first intron of the PDGF-A chain gene has suppressor activity, and that a large region, in which at least 2 nuclear proteins were directly bound, was needed for this suppressor activity. *In vivo* also, this fragment suppressed the

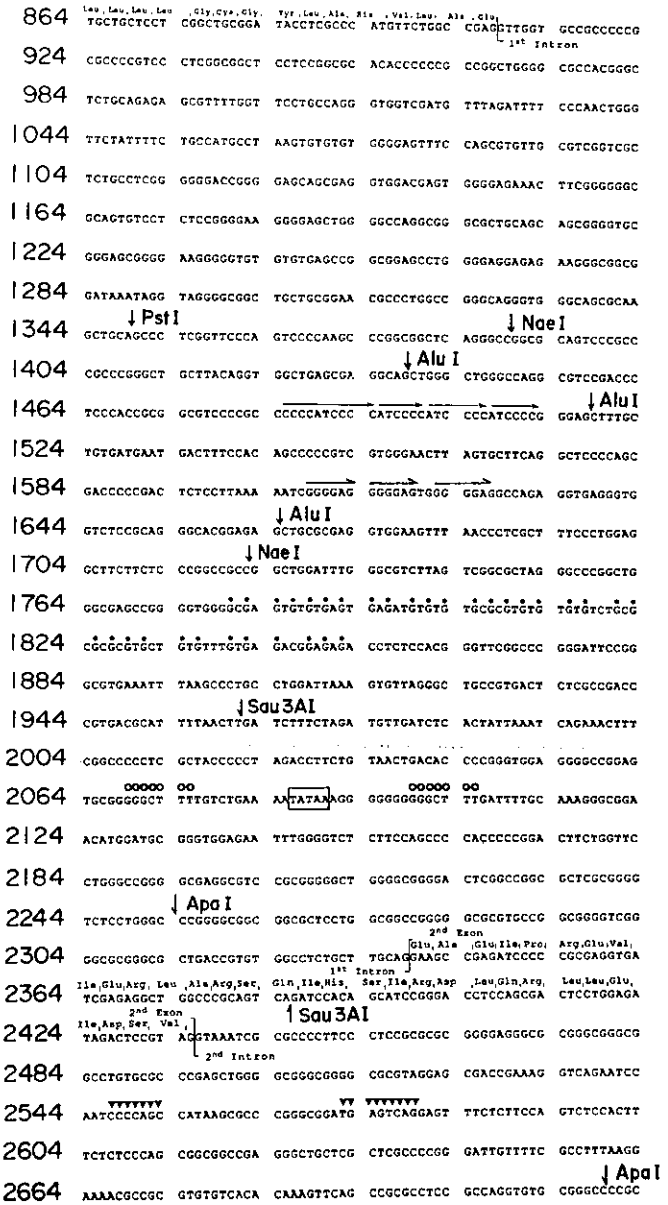

Fig. 8. Structures of the first and second introns of the PDGF-A chain gene. Direct repeat (→), alternative purine/pyrimidine sequence (●), AP1, AP2 consensus binding sequence (▼), NF-KB/H2TF1 consensus binding sequence (○), TATAA sequence (□).

expression of the PDGF-A chain via nuclear proteins. Since the fragment also suppressed the PDGF-B promoter, it may be involved in a suppression mechanism common to the PDGF-A and -B chains, and since this suppression was orientation-independent, the fragment was considered to act as a silencer.

Four tandem repeats of CCCCAT(CCCC) and three direct repeats GGGGAG were observed in this region (Fig. 8), and these short repeat sequences may act as regulators of transcription.[20–22] Collagen II gene is regulated tissue-specifically, in part through a silencer in the 5′-upstream region which suppresses an enhancer in the first intron.[23] Interestingly, a sequence identical to the short repeat sequence CCCCATCC, which we observed in the N-N fragment, was noted as one of the 5′ silencer elements.[23] The same sequence has also been found in the CRI gene[24] and thymidine kinase gene[14] (CCCCTCCCCATC), but that sequence of the thymidine kinase gene, in contrast, has enhancer activity.

The N-S fragment and S-A fragment had positive activities towards the SV40 promoter in RD cells. An alternative purine/pyrimidine sequence is present in the N-S fragment (Fig. 8). This sequence has the potential to form a left-handed conformation of the double helix (Z-DNA)[25] and has often been observed in enhancer sequences.[26] However, the N-S fragment has a slight suppressive effect, and the S-A fragment has little effect, on CAT, Xh-S activities. Gel retardation assay and photo-affinity labeling suggested the presence of proteins that bind to this region, but many of the Z-DNA binding proteins have been suggested to be phospholipid-binding proteins.[27] Further studies are needed to clarify whether the proteins observed in this study are functional in the expression of the PDGF-A chain.

In general, a suppressor element distant from the promoter exhibits its activity by inhibiting the binding of positive control factors to a neighboring enhancer.[28, 29] Unlike this mechanism, the N-N fragment in the first intron of the PDGF-A chain is considered to suppress the 5′ region by a looping model[30] via nuclear proteins bound to it. The presence of cell type-specific regulatory elements was also recently reported in the first intron of the PDGF-B chain.[6]

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