J. Biochem. Mol. Biol. Vol. 29, No. 5 pp. 468~473 September 30, 1996

Analysis of the MVM P38 Promoter Distal DNA *cis*-Elements Responsible for Transactivation by Nonstructural Proteins

Yoo-Nha Kim and Jeong Keun Ahn*

Department of Microbiology, Chungnam National University, Taejon 305-764, Korea
(Received July 26, 1996)

Abstract: The P38 promoter of minute virus of mice (MVM) is a very weak promoter which is strongly transactivated by viral nonstructural proteins. To analyze the upstream sequence of the P38 promoter which is responsible for the transactivation by nonstructural proteins in MVM, chloramphenicol acetyltransferase (CAT) reporter plasmids containing a series of 5′ deletion and internal deletion mutants of the P38 promoter were constructed. The wild type and mutant CAT constructs of P38 promoter were cotransfected into murine A92L fibroblast cells with a plasmid expressing viral nonstructural proteins by DEAE-dextran method. Each promoter activity was analyzed by CAT assay. As previously reported (Ahn *et al.*, 1992), the proximal DNA *cis*-elements required for transactivation of the MVM P38 promoter are GC box and TATA box. However, the analysis of 5′ deletion mutants showed that H-1 tar like sequence (MVM TAR) which is located between −143 and −122 relative to the transcription initiation site is also required for transactivation of the P38 promoter by nonstructural proteins. Interestingly, even if the MVM TAR was removed by internal deletion, the level of the transactivation is still 70% of wild type level of transactivation. We also found that, in addition to the MVM TAR motif, there are two other motifs which are similar to the MVM TAR sequence. When these TAR like motifs were further deleted, the levels of transactivation were decreased further. Taken together, the MVM TAR sequence and TAR like motifs located upstream of P38 promoter are playing an important role for the transactivation of P38 promoter by nonstructural proteins in minute virus of mice.
Key words: nonstructural proteins, MVM, MVM TAR sequence, P38 promoter, transactivation.

Minute virus of mice (MVM) is one of the autonomous parvoviruses containing a single-stranded DNA genome of 5 kilobases (Astell *et al.*, 1983). Parvoviruses are predominantly teratogenic agents which cause fetal and neonatal abnormalities by destroying specific cell populations which are rapidly proliferating during the normal course of development (Siegel, 1984; Brownstein *et al.*, 1992). In culture, prototype MVM productively infects mouse fibroblast cell lines (Merchlinsky *et al.*, 1983; Tattersall and Bratton, 1983). The transcriptional organization of MVM is relatively simple (Fig. 1). The virus contains two overlapping transcription units with two promoters, one at map unit 4 and the other at map unit 38 (Pintel *et al.*, 1983; Ben-Asher and Aloni, 1984). The promoters for these transcription units, termed P4 and P38, produce the three viral mRNAs containing common splicing sites between map unit 46 and 48. Two viral messages, R1 and R2, are transcribed from the P4 promoter. The R1 message class, a 4.8 kb RNA transcript, encodes one of the viral nonstructural proteins, NS-1, which is a 84 kd protein. The R2 mRNA encodes the second viral nonstructural protein, NS-2, which is a 25 kd protein. These nonstructural proteins are essential for viral replication (Cotmore and Tattersall, 1988; Naeger *et al.*, 1990; Mario *et al.*, 1992) and transactivate the P38 promoter (Doerig *et al.*, 1988; Ahn *et al.*, 1992). The R3 mRNA is transcribed from the P38 promoter and produces the viral coat proteins VP-1 and VP-2 *via* different splicing (Morgan and Ward, 1986; Clemens *et al.*, 1990). During the productive infection of virus, R1 and R2 mRNAs are transcribed at first, suggesting that the P4 promoter is an early promoter of the virus (Clemens and Pintel, 1988; Ahn *et al.*, 1989).

The P38 promoter of minute virus of mice (MVM) is a very weak promoter which is strongly transactivated by viral nonstructural proteins. The GC box and TATA box were identified as transactivation responsible DNA *cis*-elements by testing linker scanning mutants of P38 promoter (Ahn *et al.*, 1992). However, MVM nonstructural proteins are not transactivating heterologous promoter containing a GC box and a TATA box like HIV

*To whom correspondence should be addressed.
Tel: 82-42-821-6418, Fax: 82-42-822-7367.
E-mail: jkahn@hanbat.chungnam.ac.kr

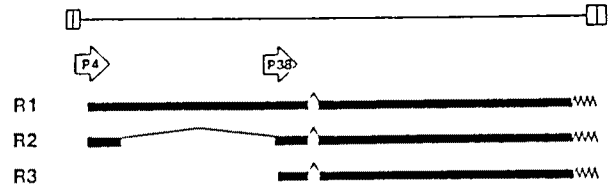

Fig. 1. The organization of the MVM genome. The top line is a diagram of the viral DNA strand terminated by the 3′ and 5′ terminal hairpin palindromes (as boxes). The position of the two promoters at map unit 4 and 38, and the transcripts of MVM, R1, R2 and R3 are aligned beneath the diagram of the viral genome.

LTR. Therefore, it is possible that there may be other DNA *cis*-elements required for the specific transactivation of P38 promoter by nonstructural proteins in addidtion to the GC and TATA box. Here we examine the distal promoter region which is upstream region of the GC and TATA box. We constructed and tested a series of 5′ deletion and internal deletion mutants of P38 promoter to identify DNA *cis*-elements required for the specific transactivation by nonstructural proteins in MVM. We found that the upstream sequence located between −143 and −122 was required for the optimal transactivation of P38 promoter by nonstructural proteins. This upstream motif, the MVM TAR sequence, is very similar to the H-1 TAR sequence which is required for the transactivation in H-1 parvovirus (Rhode and Richard, 1987; Li and Rhode, 1990). We also found that in addition to the MVM TAR sequence, there were two MVM TAR like sequences in distal MVM P38 promoter which were involved in the transactivation of P38 promoter by nonstructural proteins.

Materials and Methods

Plasmids

pKONS-1/NS-2 is the expression plasmid of viral nonstructural proteins. This plasmid was constructed by substituting the Neo gene with the MVM nonstructural protein coding sequences (nt 225-nt 3453) from pKO-neo. p38CAT plasmid contains viral sequences from nt 1085 to 2031 (*Eco*R1-*Dde*1) in front of CAT gene. Since the viral first ATG site is located at nt 2045, p38CAT plasmid contains only P38 promoter sequences which are from −920 to +26 relative to the transcription initiation site (nt 2005). pSV2CAT contains the CAT gene driven by the SV40 enhancer and SV40 early promoter. pSV0CAT is the same plasmid as pSV2CAT except that the SV40 enhancer and SV40 early promoter are removed.

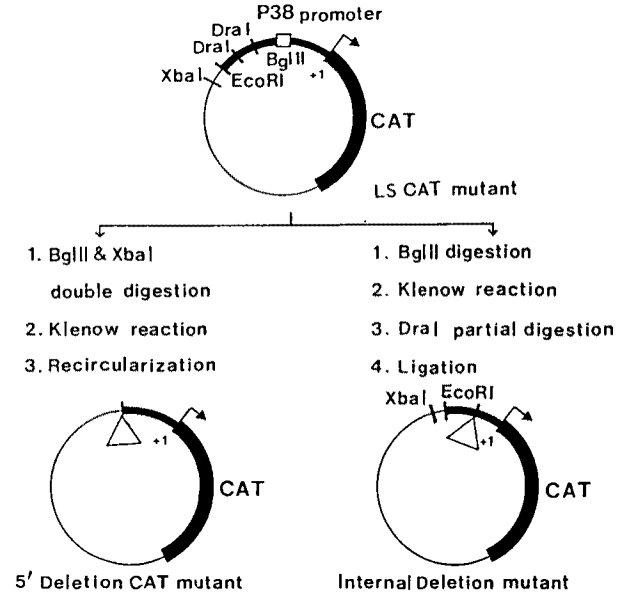

Fig. 2. Construction of 5′ deletion and internal deletion mutant CAT plasmids of P38 promoter. A series of 5′ deletion mutant and internal deletion mutant CAT plasmids were constructed by using linker scanning mutant CAT plasmids. Detailed procedures are described in Materials and Methods.

Construction of 5′ deletion mutant CAT plasmids of P38 promoter

Linker scanning mutant (LS) CAT plasmids (Ahn *et al.*, 1992) were used to construct 5′ deletion mutant CAT plasmids of P38 promoter (Fig. 2). In each LS CAT plasmid, 10 bp of P38 promoter sequence is substituted with 10 bp of *Bgl*II linker. Each LS CAT plasmid was linearized with *Bgl*II and *Xba*1 to remove the upstream promoter sequence which is upstream of *Bgl*II linker site. The large *Bgl*II/*Xba*1 fragment was blunt ended by Klenow reaction and recircularlized to construct 5′ deletion mutant CAT plasmids of P38 promoter.

Construction of internal deletion mutant CAT plasmids of P38 promoter

To construct internal deletion mutants devoid of the MVM TAR sequence in P38 promoter (pID1/3CAT), LS-163/-154 CAT and LS-137/-127 CAT were digested by *Bgl*II and *Xba*1 (Fig. 2) A DNA fragment containing promoter sequence from LS-137/-127 CAT and a fragment of CAT gene from LS-163/-154 CAT were ligated together to make pID1/3CAT. To construct the other internal deletion mutants, LS-137/-127 CAT was digested by *Bgl*II and subsequently digested partially with *Dra*1. Partially digested DNA fragments were isolated from an agarose gel and ligated after Klenow reaction.

Transfection assay

Plasmid DNAs were introduced into mouse fibroblast A92L cells by DEAE-dextran transfection method (Lopata *et al.*, 1984). 5×10^5 A92L cells per 60 mm tissue culture dish were plated in 5 ml of Dulbecco modified Eagle medium with 10% fetal calf serum (10% DMEM). After incubation for 24 h in CO_2 incubator, 2 μg of each CAT plasmid and 0.5 μg of pKONS-1/NS-2 plasmid were ethanol precipitated and resuspended in 20 μl of Tris-buffered saline (TBS). The medium was removed and the tissue culture plate was washed with phosphate-buffered saline (PBS). 2 ml of 10% DMEM was added to the plate. 60 μl of warm DEAE-dextran (10 mg/ml) in TBS was added to the 20 μl DNA solution and the DNA/DEAE-dextran mixture was added dropwise to each plate. The plates were incubated for 4 h and the DNA/DEAE-dextran/10% DMEM solution was removed from the plates. Cells were shocked by adding 2 ml of 10% dimethyl sulfoxide (DMSO) in PBS for 1 min. DMSO was removed, the plates were washed with PBS and 5 ml of 10% DMEM was added to each plate. 48 h after adding DNA/DEAE-dextran, the cells were washed with PBS. After adding 1 ml of TEN solution (40 mM Tris-Cl pH 4.5, 1 mM EDTA pH 8.0, 150 mM NaCl), cells were scraped off. Cells were spun down and resuspended in 50 μl of Tris buffer. Cells were frozen in dry ice/ethanol for 5 min and thawed 5 min at 37℃. Freeze-thaw steps were repeated three additional times. Cells were spun down for 3 min at 4℃ and supernatant was saved for cell extracts. The protein concentration of each extract was determined by the modified Bradford method (Bradford, 1976).

CAT assay

The CAT assay was carried out as previously described (Ahn *et al.*, 1992). Briefly, 20 μl of cell extract was added to 130 μl of cocktail containing 1 μl of C^{14}-chloramphenicol (50 mCi/mmol), 3 μl of 40 mM acetyl CoA, 32.5 μl of 1 M Tris-Cl pH 7.5, and 93.5 μl of ddH_2O. The reaction mixtures were incubated for 1 h at 37℃. The reaction mixtures were extracted with ethyl acetate and resuspended with 20 μl of chloroform. Samples were spotted on a silica thin layer chromatography plate (Whatman PESILG). The plates were developed with 19:1 chloroform-methanol (vol/vol). After air drying, the plates were placed on X-ray film for autoradiography.

Results and Discussion

To identify the specific responsive DNA *cis*-elements for transactivation of P38 promoter by nonstructural proteins, 5′ deletion and internal deletion mutant CAT plasmids were constructed and tested by transient transfection experiment followed by CAT assay. p38CAT plasmid contains wild type P38 promoter in front of CAT gene (Fig. 3). LS CAT plasmids are the same as p38CAT plasmid except the mutated 10 bp regions of the promoter which are substituted by 10 bp *Bgl*II linkers.

As previously reported (Ahn *et al.*, 1992), the P38 promoter is a very weak promoter and it is strongly transactivated in the presence of viral nonstructural proteins (Fig. 4, lanes 4, 5). There are a GC box, a TATA box and H-1 TAR like sequence in P38 promoter (Fig. 3). H-1 TAR like sequence is very similar to H-1 TAR which was identifed as a DNA *cis*-element for transactivation in H-1 parvovirus (Rhode and Richard, 1987). Therefore, we decided to call H-1 TAR

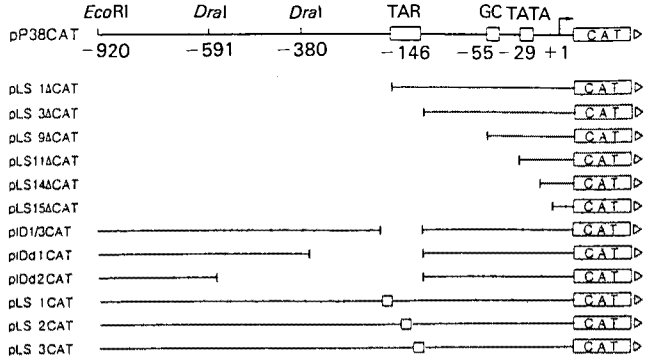

Fig. 3. Schematic representation of P38 promoter mutants. The top line is a diagram of the P38 promoter (from *Eco*R1 site to *Dde*1 site) in front of CAT gene. The boxes on the line denote the MVM TAR sequence, GC box and TATA box. The arrows indicate the major transcription initiation site (nt 2005). A series of 5′ deletion, internal deletion and linker scanning mutants of P38 promoter are aligned.

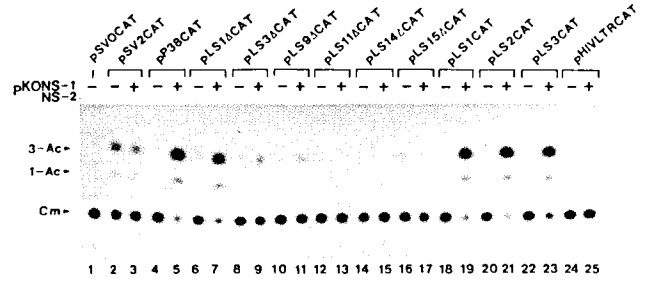

Fig. 4. Autoradiogram of CAT assay for the analysis of transactivation of 5′ deletion mutants of P38 promoter by nonstructural proteins. Each reporter CAT plasmid of 2 μg was cotransfected into A92L cell with pKONS-1/NS-2(−, no pKONS-1/NS-2; +, 0.5 μg of pKONS-1/NS-2). Cm: chloramphenicol; 1-Ac: 1-acetyl chloramphenicol; 3-Ac: 3-acetyl chloramphenicol. pSV0CAT is a negative control plasmid and pSV2CAT is a positive control plasmid.

like sequence of MVM the MVM TAR sequence. All the LS mutants scanned from −163 to +10, except the mutants of GC box and TATA box, were transactivated by nonstructural proteins, suggesting that only the GC box and TATA box are required for the transactivation of P38 promoter by nonstructural proteins (Ahn *et al.*, 1992). However, MVM nonstructural proteins are not transactivating heterologous promoter containing a GC box and a TATA box like HIV LTR (Fig. 4, lanes 24, 25).

Here, we report other DNA *cis*-elements of P38 promoter responsive for the transactivation. A series of 5′ deletion CAT mutants of P38 promoter were constructed by using LS CAT plasmids. A new DNA *cis*-element of P38 promoter was identified by the analysis of 5′ deletion CAT mutants of P38 promoter. pLS1 CAT, whose P38 promoter region is deleted up to −146 relative to the transcriptiponal initiation site, was transactivated at the wild type level by viral nonstructural proteins (Fig. 4, compare lanes 6, 7 with lanes 4, 5). This 5′ deletion mutant contains the GC box, the TATA box and H-1 TAR like sequence (MVM TAR). Surprisingly, pLS3ΔCAT, which has deletion up to −127, was not transactivated at the wild type level by nonstructural proteins (Fig. 4, lanes 8, 9). This 5′ deletion mutant contains the GC box and the TATA box without the MVM TAR motif. The transactivation level of pLS3ΔCAT is about 30% of wild type level of transactivation. In addition, pLS9ΔCAT (deletion to −72), which contains only the GC box and TATA box, showed 20% of wild type level of transactivation (Fig. 4, lanes 10, 11). pLS11ΔCAT which contains only the TATA box, pLS14ΔCAT and pLS15ΔCAT which are devoid of the GC box and TATA box, were not transactivated by nonstructural proteins (less than 10% of wild type of transactivation. Fig. 4, lanes 12-17). This suggests that the GC box and the TATA box are required DNA *cis*-elements, but not sufficient *cis*-elements for the transactivation of P38 promoter and the MVM TAR motif, which is similar to H-1 TAR sequence, is also required for wild type level of transactivation of P38 promoter by nonstructural proteins.

However, as reported previously (Ahn *et al.*, 1992), the linker scanning mutants substituting parts of H-1 TAR like sequence (MVM TAR) were transactivated in the presence of nonstructural proteins suggesting that the MVM TAR motif is not required for the transactivation of P38 promoter. Therefore, we also tested pLS1 CAT, pLS2CAT and pLS3CAT which are the linker scanning mutants which substitute 10 bp of viral sequence around the MVM TAR sequence. MVM TAR sequence is 22 bp sequence located between −143 and −122. pLS1CAT, which substitute the 10 bp upstream region of the MVM TAR sequence, showed almost same transactivation level as the p38CAT which contains the wild type P38 promoter. pLS2CAT which alter the front part of the MVM TAR sequence was transactivated at the wild type level (Fig.4, lanes 20, 21). pLS3CAT substituting the middle part of the MVM TAR sequence was also transactivated at the wild type level (Fig.4, lanes 22, 23). By testing the linker scanning mutants which change parts of the MVM TAR sequence, we were obligated to conclude that the MVM TAR sequence is not required for the transactivation of P38 promoter by nonstructural proteins. These data of linker scanning mutant were completely different and contrasted with 5′ deletion data in the function of the MVM TAR sequence.

How can we understand these conflicting results of the function of the MVM TAR sequence on the transactivation of P38 promoter by nonstructural proteins? It may be possible that these different results have come from different assay systems, namely, one is from the 5′ deletion mutants and the other from the linker scanning mutants. However, we could not rule out the possibility that there may be the other DNA *cis*-elements which are replacing the MVM TAR function of transactivation, locating in upstream of the MVM TAR sequence.

We constructed an internal deletion mutant, pID1/3CAT, which removes the entire MVM TAR sequence (Fig. 3). Interestingly pID1/3CAT was transactivated by nonstructural proteins at the wild type level (Fig. 5, lanes 9, 10), suggesting that the MVM TAR is not required for the transactivation of P38 promoter by nonstructural proteins. This result gives the same conclusion as that from the linker scanning mutants and it is counter to the result from 5′ deletion mutants. Therefore, we tested the possibility of other DNA *cis*-elements located upstream of the MVM TAR sequence which substitute the function of the MVM TAR.

We constructed two other internal deletion mutants by digesting the LS-137/-127 CAT plasmid with *Bgl*II and *Dra*1 partially. pIDd1CAT is an internal deletion muant which removes the sequence between −387 and −116. pIDd1CAT showed about 50% of wild type transactivation level (Fig. 5, lanes 13, 14). pIDd2CAT is an internal deletion mutant which removes 480 bp sequence between −598 and −118. The level of transactivation of pIDd2CAT was 30% of wild type level (Fig. 5, lanes 11, 12). Interestingly, there are two DNA motifs similar to the MVM TAR sequence in each of the deleted regions (Fig. 6). One motif (MVM TAR like motif 1) is located between −178 (nt. 1827) and −157 (nt. 1848). The other one (MVM TAR like motif 2) is located between −495 (nt. 1510) and −474 (nt.

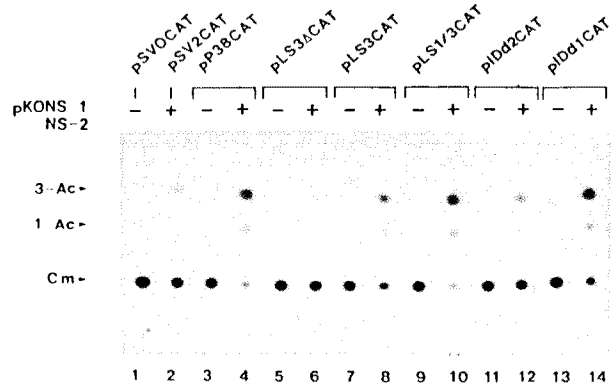

Fig. 5. Autoradiogram of CAT assay for the analysis of transactivation of linker scanning mutants and internal deletion mutants of P38 promoter by nonstructural proteins. Each reporter CAT plasmid of 2 μg was cotransfected into A92L cell with pKONS-1/NS-2 (−: no pKONS-1/NS-2; +: 0.5 μg of pKONS-1/NS-2). Cm: chloramphenicol; 1-Ac: 1-acetyl chloramphenicol; 3-Ac: 3-Acetyl chloramphenicol.

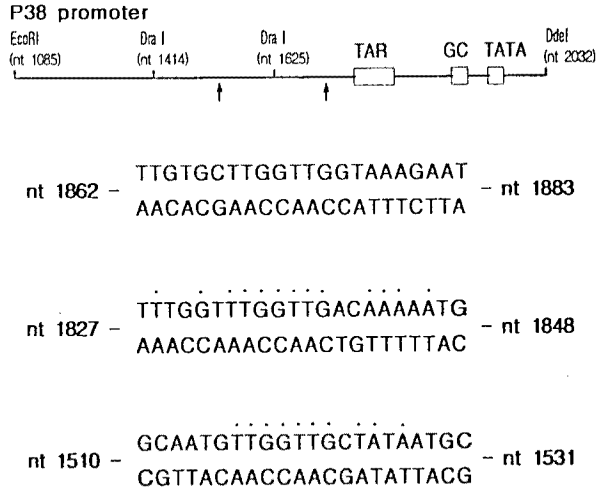

Fig. 6. Comparison between the TAR sequence and TAR-like sequence. The MVM TAR sequence and two MVM TAR-like sequences are denoted beneath the diagram of the P38 promoter. The nucleotides which are the same as the TAR sequence are indicated by dots on the TAR-like sequence. The arrows mark the positions of TAR-like motifs.

1531). The sequence comparison of these motifs with the MVM TAR motif showed the consensus sequence which is 5′ TTGGTTG 3′/3′AACCAAC5′ (Fig. 6).

Now we can understand the conflicting results of the analysis of linker scanning mutants and the analysis of 5′ deletion mutants in terms of the function of the MVM TAR sequence. In linker scanning mutants which substitute the MVM TAR sequence (pLS2CAT and pLS 3CAT), there are two additional MVM TAR like motifs located upstream of *Bgl*II linker substituting the MVM TAR motif. These two MVM TAR like motifs may play a similar role as the MVM TAR motif in the transactivation of P38 promoter by nonstructural proteins when the MVM TAR sequence is removed. These two MVM TAR like motifs may be additional DNA *cis*-elements to guarantee the transactivation of P38 promoter by nonstructural proteins, when the MVM TAR motif is not working properly. However, the MVM TAR motif is able to work as a DNA *cis*-element along with the GC and TATA boxes without help of two MVM TAR like motifs, since 5′ deletion mutant containing only the MVM TAR along with the GC and TATA box was transactivated at the wild type level of transactivation (pLS1ΔCAT). Therefore, the DNA *cis*-elements required for the transactivation of P38 promoter by nonstructural proteins are the MVM TAR motif, the GC box, the TATA box and two MVM TAR like motifs which can substitute the function of the MVM TAR motif.

We do not think that viral nonstructural proteins are interacting with the GC box and the TATA box directly. We believe that viral nonstructural proteins may interact with general transcriptional complex bound on the TATA box and GC box to transactivate the P38 promoter. It may be possible that MVM viral nonstructural proteins can bind to the MVM TAR motif and two additional MVM TAR like motifs. Recent experiments showed that viral nonstructural protein NS-1 is bound to the sequence 5′ ACCAACCA 3′ in the replication origin of single stranded MVM genome (personal communication with Cotmore and Tattersall, unpublished data). The MVM TAR core sequence is very similar to this sequence suggesting that viral nonstructural proteins may bind to the MVM TAR motif. Currently we are working on the interactions between viral nonstructural proteins and the MVM TAR sequence.

Acknowledgement

This paper was supported by NON-DIRECTED RESEARCH FUND, Korea Research Foundation, 1994.

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