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Isolation of CD4 Genomic Clones and Role of Its 5′ Upstream Region in CD4 Expression

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Three clones containing mouse CD4 gene were prepared using AKR genomic cosmid library. The role of 6,500 bp 5′ flanking region of the first exon of the AKR CD4 gene in tissue or developmental stage specific expression of CD4 has been studied. The deletion constructs containing various amounts of CD4 5′ flanking sequences were prepared, and they were transfected into the cell lines representing different cell types or developmental stages of CD4 expression. Study of the reporter gene expression revealed that at least 1,700 bp of 5′ flanking region did retain promoter activity for CD4 expression. This area did not seem to contain enhancer activity for a full expression of CD4. However, the putative promoter interacted with other tissue specific enhancer sequence and showed the tissue specificity of the enhancer element.

KEY WORDS □ mouse CD4, gene expression, promoter

The mouse CD4 (L3T4) is a cell surface glycoprotein expressed on thymocytes and on a specific population of T lymphocytes (1). It is traditionally used as a marker to divide T cell population into two subsets along with CD8 (2). CD4 expressing T lymphocytes have functions of helper/inducer while CD8 expressing T lymphocytes represent cells with cytotoxic/suppressor functions. At the molecular level, CD4 is involved in recognizing class II MHC (major histocompatibility complex) on the antigen presenting cell. CD4 is thought to bind non-polymorphic determinant on class II molecule, and thus to increase avidity of the interaction between T cells and antigen presenting cells (3-5). Gene transfection studies support the idea that CD4 enhances T cell antigen recognition (6). However, Bank and Chess (7) showed that CD4 monoclonal antibody could block mitogen induced T cell activation. Because this inhibition of the mitogen response occurs in the absence of accessory cells expressing class II MHC, the inhibition can not be due to the failure of CD4 to bind class II MHC. This study suggests that CD4 may be involved in signal transduction process as well. The association of the CD4 with the T cell specific internal tyrosine kinase $p56^{lck}$ strongly supports this role of CD4 (8-10).

It has been suggested that CD4 and CD8 are also involved in the development of thymocytes into mature T lymphocytes (11-13). During T cell development in the thymus, thymocytes are thought to be subjected to complex selection processes. During thymic selection of the developing thymocytes, the expressions of CD4 and CD8 keep changing according to the developmental stages of thymocytes. Bone marrow precursor cells which express neither CD4 nor CD8 enter thymus and proliferate in cortical region of the thymus (14). These double negative ($CD4^-$, $CD8^-$) precursor thymocytes thought to be differentiated to double positive ($CD4^+$, $CD8^+$) thymocytes expressing both CD4 and CD8. Some of the double positive thymocytes will become mature single positive ($CD4^+$, $CD8^-$ or $CD4^-$, $CD8^+$) thymocytes. Only a fraction of these mature single positive thymocytes leave the thymus and become mature peripheral T lymphocytes (15, 16).

As described above, regulation of CD4 gene expression is very complex in that this gene exhibits tissue specific, subset specific, and developmental stage specific expression, as well as a temporal shut down of expression in CD8 cells. Unlike other genes, mouse CD4 gene possesses a long intron of 9 kb between the first exon encoding 5′ untranslated region and the second exon encoding an amino terminal domain including ATG translation start codon. To study CD4 expression, it is important to identify which area is responsible for the promoter activity. In the work reported here, the structure of 90 kb area surrounding mouse CD4 gene from AKR mice has been shown. The role of 6.5 kb 5′ flanking region of CD4 gene in tissue specific and developmental stage specific expression of CD4

has also been studied.

MATERIALS AND METHODS

Gene structure and sequence

The structure of the gene encoding mouse CD4 was determined by using three overlapping clones isolated with mouse CD4 cDNA probes (17) from a AKR liver genomic cosmid library (gift of J. Parnes, Stanford University). The nucleotide sequence of 0.7 kb 5′ flanking area was determined by the dideoxynucleotide chain termination method (18) using inserts from the region of 0.7 kb Pvu II-Kpn I (Fig. 1b) subcloned into phage M13 vectors mp18 and mp19.

Plasmid constructs

DNA fragments containing different amount of 5′ flanking region of the first exon of mouse CD4 gene were prepared using restriction enzymes and nuclease Bal 31 (19). Various DNA fragments ranging from 100 bp to 6,500 bp in size were then subcloned into the pXP2N plasmid vector (20) which contains fire-fly luciferase gene (Fig. 2). pRSVgal plasmid (21) expressing β-galactosidase was also used. The supercoiled plasmid DNA of the each construct was prepared using standard cesium chloride gradient. Quality and amount of each DNA were determined using absorbance at 260 nm and agarose gel electrophoresis.

Transfection and analysis of expression

DNA of each construct was co-transfected with pRSVgal DNA into the cell lines representing different cell types or different stages of CD4 expression by electroporation as described by the manufacturer using Bio-Rad gene pulser equipment (Bio-Rad Laboratories, Richmond, California, U.S.A.). The supercoiled plasmid DNA of the each construct corresponding to the molar concentration of 10 mg of a 9 kb plasmid was co-transfected with 10 μg of pRSVgal DNA and 10 μg of BALB/c carrier DNA. The transfection efficiency was normalized using transfections of the p0633 construct and the pRSVgal construct. Forty eight hours after transfection, cells were lysed and examined for activities of luciferase and β-galactosidase. Luciferase activities were assayed by measuring luminescence illumination as described by deWet and co-workers (22) and β-galactosidase activities were analyzed as described by Hall and co-workers (23). All transfections were performed at least in triplication. The 1010, C6VL, Eb288, and 2017 cell lines are all thymoma cell lines (24, 25). The expressions of CD4 and CD8 on these thymoma lines are;.1010 is $CD4^+$, $CD8^+$ (double positive); C6VL is $CD4^+$, $CD8^-$ (CD4 single positive); Eb288 is $CD4^-$, $CD8^+$ (CD8 single positive); 2017 is $CD4^-$, $CD8^-$ (double negative). The L1.2 and Ag8653 cell lines are transformed B cell lines (26) and they do not express neither CD4 nor CD8. The Ltk^- is a transformed endothelial line (27) and also does not express both CD4 and CD8. All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. They were tested for the expression of CD4 and CD8 using fluorescence activated cell sorter and confirmed as expected. The contamination of mycoplasma were also tested frequently.

RESULTS AND DISCUSSIONS

Structure of the gene encoding AKR CD4

The structure of the gene encoding CD4 from AKR mice was determined by restriction mapping using three overlapping clones. The organization of the 90 kb area surrounding CD4 gene is illustrated in Fig. 1a. The locations of the exons were determined by comparing the *Eco*RI, *Bam*HI and *Hin*dIII restriction map of the clone 391-11-1 with that of B10.CAS2 CD4 gene (28), and were also confirmed by hybridization with the CD4 cDNA probe (Data not shown). There is no difference in restriction map in the coding region of CD4 genes from B10.CAS2 and AKR mice. The cloned AKR CD4 gene is composed of approximately 30 kb of 5′ region, 30 kb of coding region and 30 kb of 3′ region of CD4 gene. The detailed restriction map of 6.5 kb 5′ flanking region of the first exon of AKR CD4 gene is shown in Fig. 1b. The restriction sites such as *Xba*I, *Pvu*II, *Bgl*II, *Bam*HI, *Hin*dIII, *Xho*I and *Eco*RI at 0.47 kb, 0.7 kb, 1.2 kb, 1.7 kb, 2.7 kb, 3.5 kb and 6.5 kb upstream of *Kpn*I site, respectively, were used to prepare for the deletion constructs. The *Kpn*I site inside of the first exon coding for 5′ untranslated region was also used.

Preparation of deletion constructs

There are several cis-acting elements that are required for accurate and efficient transcriptional control in eucaryotic genes. Some of those elements are a promoter region, an enhancer element and possibly a silencer element. In order to identify these cis-acting elements and their transacting factors, identification of promoter activity must be performed first. The promoter is usually located directly upstream of the gene in the 5′ flanking region and is required for initiation of transcription of the gene. However, unlike other genes, the first exon of the mouse CD4 gene does not encode a protein coding sequence, which makes the location of promoter region uncertain. Presence of a long intron between the non-protein coding first exon and the protein coding second exon provides more uncertainty. To examine the possibility that the promoter of mouse CD4 gene is in the 5′ flanking region of the first exon of CD4 gene, various constructs containing different amount of upstream sequences were prepared. Seven DNA fragments containing bigger than 470 bp of the

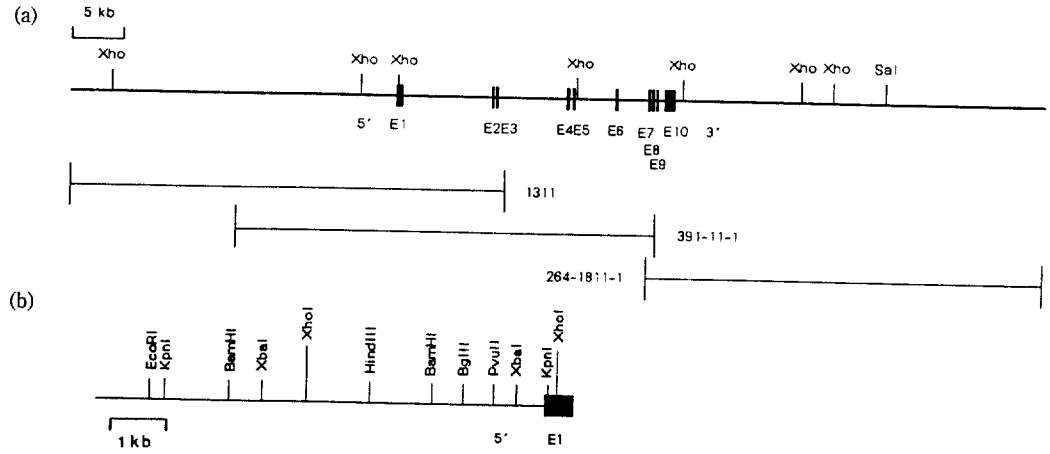

Fig. 1. *Structure of the AKR CD4 gene. (a) The exons encoding CD4 gene are indicated by black boxes and each exon is indicated by E1 through E10, respectively. 1311, 391-11-1 and 264-1811-1 represent three cosmid clones used in this mapping. Restriction endonuclease sites: Xho, XhoI; Sal, SalI. (b) Restriction endonuclease sites in 6.5 kb 5' flanking region of the first exon of AKR CD4 gene.*

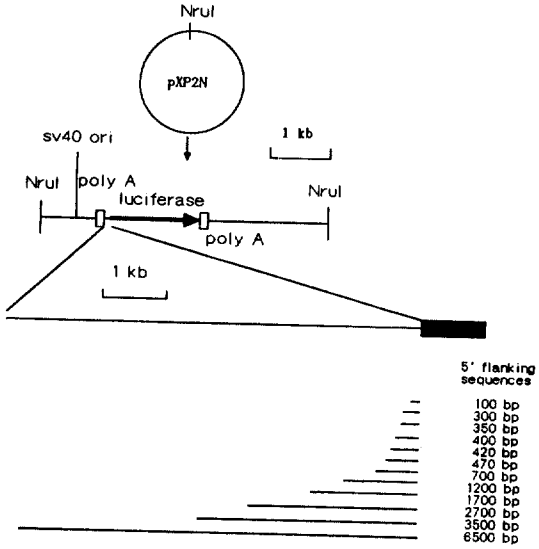

Fig. 2. *Structures of the pXP2N constructs containing different amounts of 5' flanking sequence of AKR CD4 gene. The inserts are indicated at the bottom and the numbers in the right side of them represent the distances from the KpnI site in the first exon. The orientations of the inserts are all in the same direction to that of luciferase cDNA. Construct p0633 was prepared by subcloning of 4.3 kb HindIII-SalI fragment into the same region of the pXP2N plasmid. The 4.3 kb fragment contains both enhancer and promoter activities of human b-actin gene (29). This p0633 construct was used as a positive control for the luciferase expression.*

5' flanking region were prepared using restriction endonuclease, and five DNA fragments containing 100 bp, 300 bp, 350 bp, 400 bp and 420 bp of the 5' flanking region were prepared using nuclease Bal 31 as described by Poncz and coworkers (19). Each of the fragment includes *Kpn*I site in 5' untranslated exon of CD4 gene at the 3' end of the fragment (Fig. 1b). These fragments then were subcloned into polylinker region between the poly A addition site and the luciferase reporter gene in the pXP2N plasmid as shown in Fig. 2. Restriction endonuclease study showed that the orientations of all inserts were in the same direction to that of the luciferase gene (Data not shown). Roles of these upstream fragments in CD4 expression have been studied using fire-fly luciferase as a reporter gene. pXP2N is an SV40 derived vector and contains two poly A addition site at 5' and 3' of the luciferase cDNA lacking its own promoter. Transfection of this pXP2N vector alone did not produce any luciferase expression in various cell lines (data not shown) probably due to the effective blocking of non-specific transcription by upstream poly A addition site. As a positive control construct, a DNA fragment containing the enhancer and the promoter activities of human β-actin gene (29) was subcloned into the same polylinker region (p0633), and showed high amount of luciferase expressions when they were transfected into the cell types used in this study (Table 1).

Expression of CD4

DNAs of the pXP2N constructs containing different amount of 5' flanking sequences were co-transfected into various cell lines with pRSVgal plasmid as described in Materials and Methods.

Table 1. *Expression of the luciferase reporter gene in the transfection of various cell lines. Each number represents average luciferase/β-galactosidase activities, and is a relative number to that of p0633 transfection which is set as 100 arbitrarily. pXP2N represents pXP2N vector without any inserts and the numbers of base pairs in the left most column describes the size of inserts in pXP2N vector. The CD4 expressions in the cell lines used are: 1010 and C6VL are CD4⁺; other cell lines are CD4⁻. nd; not determined.*

	L1.2	Ag8653	EB288	C6VL	2017	1010	Ltk
pXP2N	0	0	0	0	0	0	2
100bp	0	0	0	0	0	0	nd
300bp	nd	3	2	2	0	1	nd
350bp	nd	2	1	3	0	2	nd
400bp	nd	5	2	4	0	1	nd
420bp	nd	2	4	2	1	2	nd
470bp	3	4	3	4	1	3	19
700bp	nd	1	7	2	0	3	nd
1200bp	nd	2	6	3	0	3	nd
1700bp	nd	2	8	3	0	2	nd
2700bp	2	1	6	5	0	5	26
3500bp	3	2	9	2	1	4	nd
6500bp	nd	4	7	4	0	3	nd
p0633	100	100	100	100	100	100	100

The pRSVgal plasmid was used as an internal control to compensate experimental variations since it is expressing β-galactosidase constitutively. In order to compare the effects of inserts on reporter gene expression, the luciferase activity of the lysate from each transfection was divided by β-galactosidase activity. The luciferase/β-galactosidase value of the lysates from the transfection with the p0633 construct was used as a positive control, and is regarded as a full expression. Luciferase/β-galactosidase values of the lysates from the other transfections are expressed as the relative numbers to that of p0633 as 100.

Table 1 shows luciferase/β-galactosidase of each transfection in various cell lines. As expected, the lysate from the transfection with pXP2N vector does not show any significant expression of luciferase. Transfections with the constructs containing bigger than 300 bp 5′ flanking sequence show a little increase in luciferase expression compare to that of a negative control. The construct containing 100 bp insert did not show any increase of the reporter gene expression. However, all transfections into either CD4 positive cell lines (C6VL, and 1010) or CD4 negative cell lines (others) did not show significant increase in luciferase activities compare to that of p0633. It is presumed that both promoter and enhancer

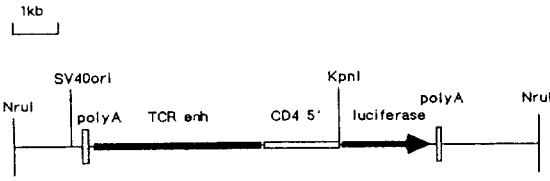

Fig. 3. *Structure of p0982 construct containing enhancer element of the T cell receptor β chain gene. A 3.8 kb BamHI fragment from the 3′ region of T cell receptor β chain gene (30) was subcloned into the construct containing 1700 bp of 5′ flanking region. This 3.8 kb fragment is shown to possess only an enhancer activity without any promoter activity. The CD4 5′ represents 1700 bp 5′ flanking region of the first exon of AKR CD4 gene.*

Table 2. *Expression of luciferase reporter gene in the transfection of p0982. Each number represents average luciferase/β-galactosidase activities on duplicated assays, and is a relative number to that of p0633 transfection which is set as 100 arbitrarily. Eb288, C6VL, 2017 and 1010 are T cell lines while Ag8653 is a B cell line*

	Ag8653	Eb288	C6VL	2017	1010
pXP2N	0	0	0	0	0
100bp	0	0	0	0	0
470bp	2	4	3	2	2
1700bp	3	7	2	1	3
p0633	100	100	100	100	100
p0982	5	324	624	798	832

elements may be required for the full expression of CD4 gene, and the area studied here does not seem to contain both elements. However, a little increases of reporter gene expression in the transfections of the constructs containing bigger than 300 bp flanking region suggests that this area could possibly possess a putative promoter element. Although it is not expressing CD4 and CD8 on cell surface, Ltk⁻ cells shows significant numbers in transfections of constructs containing 470 bp and 2700 bp 5′ flanking sequences. It is hard to tell why transfections in Ltk⁻ cell show significant expression of the reporter gene at this moment, but the data imply indirectly that the low expression in other cell lines is not due to the defects in our deletion constructs. The low numbers obtained in transfection of 2017 cell lines seems to be due to its very low transfection efficiency.

In order to confirm whether 5′ flanking region of the first exon of CD4 gene contains a promoter element, another construct (p0982) was prepared.

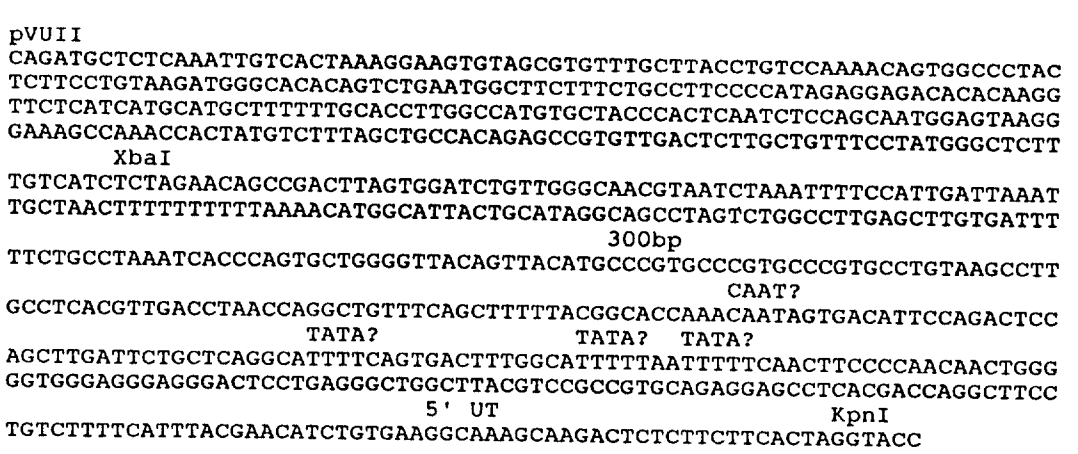

Fig. 4. *The nucleotide sequence of the 0.7 kb 5' flanking region of AKR CD4 gene. KpnI, XbaI and PvuII represent the KpnI, XbaI and PvuII restriction endonuclease sites, respectively. 5' UT shows 5' end of the first exon which codes for 5' untranslated region and 300 bp indicates 300 bp region from the KpnI site in the first exon. The putative TATA and CAAT boxes are also indicated.*

As shown in Fig. 3, a 3.8 kb fragment containing enhancer element of the T cell antigen receptor β chain gene (30) has been subcloned into construct containing 1700 bp of 5' flanking region. This 3.8 kb fragment is shown to possess only an enhancer activity without any promoter activity. If the area we tested contains at least a promoter element, this enhancer element should increase the reporter gene expression from the promoter as high as that of the transfection with p0633 which is used as a positive control. If the area we tested does not possess a promoter activity, transfection with this construct will not give any significant increase in luciferase activity. The transfections of thymocyte lines (C6VL, 1010, Eb 288, and 2017) show three to eight folds increase in luciferase activities than those of the p0633 transfections as shown in Table 2. The increases in reporter gene expression observed in the transfections of CD4 non-expressing thymocytes as well as CD4 expressing thymocyte lines. Since T cell antigen receptor β chain gene is expressed in most of T cells, these results are expected. However, there were no increase in luciferase activity in the transfections of Ag8653 which is a B cell line. This result supports that the assumption that the area studied here may possess promoter activity and the low expression of reporter gene may be due to the lack of enhancer element. It is not possible to know whether this promoter does possess tissue specificity at this moment due to the low expression. However, this result shows that the promoter responds to tissue specific enhancer element (at least in the case of a construct containing 1700 bp 5' flanking sequence) and shows up the tissue specificity of the enhancer element. Since the putative CD4 enhancer may interact with CD4 promoter, it is possible to use this construct to screen the enhancer activity using the constructs containing CD4 promoter and various inserts from the 5' region, the coding region and the 3' region of the AKR CD4 gene.

To identify whether 5' flanking region of AKR CD4 gene possesses necessary elements for a promoter activity, the 700 bp area of 5' flanking region was sequenced. As shown in Fig. 4, there are TATA like sequences and a CAAT like sequence as in other promoters (31). These TATA and CAAT boxes are located within the 300 bp flanking region of the first exon and located about 40 to 60 bp apart each other. The report that the transcription initiation site of mouse CD4 gene located near the 5' end of the first exon (28) suggests that these boxes can be necessary elements for CD4 gene transcription. Several DNase I hypersensitivity sites were also noticed in this area (data not shown) and these observations imply that at least 1700 bp , possibly 300 bp, 5' flanking area of the first exon of mouse CD4 gene possess a promoter activity.

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초 록: CD4 유전자의 분리 및 그 유전자의 5′ 지역이 CD4의 발현에 미치는 영향
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생쥐의 CD4 유전자를 AKR genomic cosmid library를 이용하여 분리 하여, 이들로부터 CD4 유전자의 5′ 상류 6,500 bp 지역이 CD4의 조직 특이적 그리고 분화 단계 특이적 발현에 미치는 영향을 조사 하였다. CD4 유전자의 첫번째 exon의 상류 6,500 bp 지역으로 부터 여러가지 deletion construct들을 만들어, 이들을 CD4 발현의 여러단계를 나타내는 세포주에 transfection 하였다. 이들 transfection된 세포들로부터 발현되는 reporter 유전자의 양을 측정한 결과, 적어도 약 1,700 bp 상류 지역내에 CD4의 promoter 활성이 존재하는 것으로 나타났다. 상류 6,500 bp내에 CD4의 충분한 발현에 필요한 enhancer 기능이 있는 것으로 보이지는 않았으나, 이 promoter 활성이 다른 조직 특이적 유전자의 enhancer와 상호 작용을 할 수 있는 것으로 나타났다.