

## ORIGINAL PAPER

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## The genomic organization of *NKG2C*, *E*, *F*, and *D* receptor genes in the human natural killer gene complex

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**Abstract** Interactions of natural killer cell receptors with their cognate ligands play a major role in regulating NK cell function. The *NKG2* gene family encodes several highly similar proteins, which are known to form heterodimers with the CD94 receptor. These dimers play a role in the inhibition as well as the activation of NK cells. We have analyzed the gene structures of the *NKG2C*, *D*, *E*, and *F* genes, and determined their genomic organization. Restriction mapping and sequencing revealed the four genes to be closely linked to one another, and of the same transcriptional orientation. An exon duplication within the *NKG2C* and *E* genes was identified, although the duplicated version of this exon has not yet been found in mRNA sequences. The *NKG2C*, *E*, and *F* genes, despite being highly similar, are variable at their 3' ends. We show that *NKG2C* consists of six exons, whereas *NKG2E* has seven, and the splice acceptor site for the seventh exon occurs in an *Alu* repeat. *NKG2F* consists of only four exons and part of exon IV is in some cases spliced to the 5' end of

the *NKG2D* transcript. *NKG2D* has only a low similarity to the other *NKG2* genes.

**Key words** Natural killer cells · *NKG2* · NK complex · Genomic organization · Chromosome 12

### Introduction

Natural killer (NK) cells constitute a distinct lineage of lymphocytes which are known to mediate lysis of certain tumour- and virus-infected cells (Trinchieri 1989). A major NK cell function is to lyse cells that have lost or have a significantly reduced level of expression of self major histocompatibility complex (MHC) class I molecules. This cytolytic function is controlled by a balance of activatory and inhibitory signals which are transduced by specific NK-cell receptors (Biassoni et al. 1996; Höglund et al. 1997; Moretta et al. 1997; Yokoyama and Seaman 1993).

Several NK cell receptor genes have been cloned and sequenced (Raulet and Held 1995). In humans, these genes were found to be contained in two different superfamilies: the *p50/p58/p70* genes (Colonna and Samaridis 1995; d'Andrea et al. 1995; Pende et al. 1996; Wagtmann et al. 1995) are members of the immunoglobulin-like (Ig-like) superfamily. The second group of receptors comprises the *NKG2* family (Adamkiewicz et al. 1994; Hofer et al. 1992; Houchins et al. 1991), *NKR-P1* (Lanier et al. 1994) and *CD94* (Chang et al. 1995), all of which are type II transmembrane proteins with a C-type lectin domain. Four closely related transcripts have been identified belonging to the *NKG2* family: *NKG2A* and *B* which are alternative splice variants, *NKG2C*, *NKG2E*, and recently *NKG2F* (Plougastel and Trowsdale 1997). *NKG2D* (Houchins et al. 1991) is related but displays only a low sequence similarity with the other *NKG2* members. It has recently been shown that a *CD94/NKG2A* heterodimer is involved in MHC class I recognition leading to NK-cell inhibition (Braud et al. 1998; Brooks et al. 1997; Carretero et al. 1997;

The nucleotide sequence data reported in this paper have been submitted to the EMBL/ Genbank nucleotide sequence databases and have been assigned the accession numbers AJ001683–AJ001689

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Lazetic et al. 1996). CD94/NKG2C heterodimers have also been described (Lazetic et al. 1996) and are thought to exhibit an activatory rather than inhibitory function (Duchler et al. 1995; Houchins et al. 1997).

The mouse *Nkrp1* and *Ly49* gene families are genetically linked in a region termed the natural killer gene complex (NKC) on distal mouse chromosome 6 (Yokoyama and Seaman 1993). Recently a 2 Mb yeast artificial chromosome (YAC) contig and physical map of the mouse NKC has been established (Brown et al. 1997), which encompasses the *Nkrp1* family, *Cd69* and the *Ly49* cluster of genes. Sequences for mouse *CD94* and *Nkg2d* have also recently been identified (Vance et al. 1997). Rat homologues for these latter two genes have been mapped to the rat NKC on chromosome 4 (Dissen et al. 1996; Dissen et al. 1997). The homologous human region on the short arm of chromosome 12 is now being characterized. Recently *CD94* and the *NKG2* family were localized in a YAC contig in the direct neighborhood of the STS marker *DI2S77* in 12p13 (Renedo et al. 1997). In addition, the exon/intron struc-

ture of *NKG2A/B* has recently been reported (Plougastel et al. 1996).

We analyzed the gene structures of the *NKG2C*, *D*, *E*, and *F* genes. *NKG2F* was independently isolated in this study and also recently reported by Plougastel and Trowsdale (1997). We found that the four genes are closely linked on chromosome 12 within one P1-derived artificial chromosome (PAC) clone. Comparison of these genes provides an insight into the evolution of this gene family.

## Materials and methods

### Probes, primers, libraries, and cell lines

A human PAC library (Ioannou et al. 1994; Lehrach et al. 1990) was obtained from the German Resource Center. An oligo dT-primed cDNA library generated from purified NK cells from four distinct donors was constructed using pCDM 8 (G. Freeman and E. Vivier, unpublished). Primers for the amplification of *NKG2C*, *E*, *F*, and *D* are shown in Table 1. A 1.2 kb *Xba* I / *Eco* RI

**Table 1** Primers

Name	Sequence (5'-3')	Temp.	Gene	Position
RDYAC 5	CCTCCCTGAATCATCAAGG	56	<i>C</i>	Exon I (for)
RDYAC 11	TCTGCGTCTTGTATTCGG	56	<i>C</i>	Exon III (rev)
RDYAC 33	CTATCTTTGAAAGCCAAACC	60	<i>C</i>	Exon V (rev)
RDYAC 32	CTGGAGCAGAACAAATTCTCC	60	<i>C</i>	Exon III (for)
RDYAC 43	CTTTATTGAAGTGTTCATGTACAAAGC	60	<i>C</i>	Exon VI (rev)
RDYAC 42	GATTGGTGTGTTTTTCGTAACAGC	60	<i>C/E</i>	Exon V (for)
NKG2-Erev	ACTGGTCTGATATAAGTCCACG	58	<i>E</i>	Exon VI (rev)
NKG2-5UTR-3	ATACAGAATACATCTAAAAAATG	52	<i>E</i>	Exon I (rev)
NKG2-5UTR-5	TCAGAGAAGCATTGTTGAAGG	52	<i>E</i>	5'-UTR (for)
E-5' (E-PAC)	GTTGTATCAGTGAGGTTCAAG	62	<i>E</i>	Intr. III (for)
E-3' (E-PAC)	CTATAGAAAGCAGACTAGAAGAG	62	<i>E</i>	Exon IV (rev)
C-3' (C-PAC)	TATCTATAGAAAGCAGACTGGAG	62	<i>C</i>	Exon IV (rev)
C-5' (C-PAC)	GTTGTGTATCAGCGATGTTCAAC	62	<i>C</i>	Intr. III (for)
E-ex6-3'	TTGATTTATTTCCAATCATAACGG	56	<i>E</i>	Exon VII (rev)
E-int6-for	CATATCTTTCTTATATTGAAGTGG	55	<i>E</i>	Intr. VI (for)
E-int6-rev	AACCAGTACCTCACCATGAGC	55	<i>E</i>	Intr. VI (rev)
D-ex3F	ATTTGAATGGGGTGGATTTCGTGGTCCGGAG	62	<i>D</i>	Exon III (for)
D-in6R	GCGAAGGTTGCATTGTGCTGAGATCACCCCA	62	<i>D</i>	Intr. VI (rev)
F-ex1 for	GATGAATAAACAAAGAGGAACCTA	50	<i>F</i>	Exon I (for)
F-ex4 rev	AGCACAGCCAGCAAACCTCTT	50	<i>F</i>	Exon IV (rev)
Dfor	TTCTGCTGCTTCATCGCTGTAG	57	<i>D</i>	Intr. IV (for)
Drev	CGGTCAAGGGAATTTGAACTTC	57	<i>D</i>	Intr. IV (rev)
D3F	CTCTTCAGGAAATGTGATTCAGG	55	<i>D</i>	Intr. IB (for)
D4R	CCACCCATTGTTGGATAAATG	55	<i>D</i>	Intr. IB (rev)
D5F	TAGAAGGCTTTTATCCACAAGAATCA	55	<i>D</i>	Exon II (for)
D6R	GCAAAGTACCCTTAGCAAAAATCC	55	<i>D</i>	Intr. III (rev)
T7	ATACCACTCACTCACTATAGGGAG	40*	-	PAC vector
SP6	CGACATTTAGGTGACACTATAG	40*	-	PAC vector
NKG2C-5'	GTGCACCGTAAACCACCTA	40*	<i>C</i>	Intr. IV (rev)
NKG2C-3'	GCCTGAAATGGCAGATAGTG	40*	<i>C</i>	Intr. V (rev)
NKG2E-5'	GTGAAAAAGACAGACATG	40*	<i>E</i>	Intr. IV (rev)
NKG2E-3'	GAATGTCTGAAATAGTAC	40*	<i>E</i>	Intr. V (rev)
NKG2F-5'	TTGTAACATATTGTTTTGC	40*	<i>F</i>	Intr. III (for)
NKG2F-3'	GAGTCCTGCCAGCAACATTA	40*	<i>F</i>	3' Exon IV (rev)
NKG2F-ex4	TGCTTCGAAGAAGACTCTGAT	42*	<i>F</i>	Exon IV (for)
NKG2D-exIB	GTTCAAATTGGCAACTTACAGCC	45*	<i>D</i>	Exon IB (rev)
NKG2D-in8F	TATGTCCTTACCTCTTTCTTTCC	50*	<i>D</i>	Intr. VIII (for)
NKG2D-in8R	GAAAACCTATTCTTAAAGAG	42*	<i>D</i>	Intr. VIII (for)

(for) and (rev) refer to forward and reverse respectively

\* Hybridization temperatures

*NKG2C* cDNA fragment and a 1.7 kilobase (kb) *Xba* I / *Eco* RI *NKG2D* cDNA fragment were derived from lambda phages (Houchins et al. 1991).

The NKL cell line (Robertson et al. 1996) was kindly provided by M.J. Robertson and grown in RPMI 1644 medium containing 10% human serum and 100 units/ml human recombinant IL2 (a gift from Sandoz, Inc., Vienna, Austria).

#### Identification of a PAC clone from the *hNKC* region

*NKG2C* was labeled by random priming (Feinberg and Vogelstein 1983). Hybridizations were performed in a modified Church hybridization buffer [Church and Gilbert 1984: 0.25 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 5% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetate] for 16 h at 65°C, and washed three times for 30 min each at 65°C: 1) 2× standard sodium citrate (SSC), 0.1% SDS, 2) 0.5× SSC, 0.1% SDS, 3) 0.1× SSC, 0.1% SDS. Filters were exposed for 3 days at -80°C.

#### Preparation of PAC clone DNA and restriction mapping

The PAC was grown in LB containing 25 µg/ml kanamycin. Mini-preparations were performed as described previously (Francis et al. 1994). The clone was sized by digesting 500 ng miniprep DNA with *Not* I (New England Biolabs, Schwalbach, Germany), and electrophoresing on a pulsed field gel (PFG, CHEF Biorad) at 14°C with ramped switch times from 4 s to 24 s and a run time of 15 h. Larger quantities of DNA were prepared using cesium chloride gradients (Sambrook et al. 1989).

*Not* I, *Apa* I, *Kpn* I, *Bst* EII, and *Asp* 718 digestions were separated by PFG electrophoresis (Pharmacia Gene Navigator with an hexagonal electrode, 250 V, switch time 4 s, run time 14 h at 9°C), and regular gel electrophoresis. To prevent restriction at dcm-methylated *Asp* 718 sites the respective digestions were carried out at 4°C. Hybridizations to Southern blots were performed at 40 to 50°C with gene-specific oligonucleotides end-labeled with T4 polynucleotide kinase and gamma (32P)-ATP (Table 1), in the presence of 0.5 mg/ml yeast tRNA, 6× SSC and 0.1% SDS. Filters were washed three times in hybridization buffer without tRNA.

#### Construction and characterization of a shotgun library from D21184

Ten µg of purified PAC DNA was sonicated to give an average insert size of 1.8 kb. End-repaired DNA was size selected and cloned into pUC18 (Pharmacia Biotech, Uppsala, Sweden). DNA was transformed in KK2186 cells (Sambrook et al. 1989), and clones were arrayed and gridded. To detect clones containing *NKG2C* and *E* sequences, PCR probes were generated using the following primer pairs: RDYAC5 and RDYAC11, RDYAC33 and RDYAC32, RDYAC42 and RDYAC43, RDYAC42 and *NKG2-E*rcv, *NKG2E-5'* UTR-5 and *NKG2E-5'*UTR-3 (Table 1). Two primer hybridizations were performed using *E-int-rev* and *NKG2E-ex* 6, which surround the *Alu* element in *NKG2E*. *NKG2F* shotgun clones were co-identified in screens with *NKG2E* and *C* probes. To complete the genomic *NKG2F* sequence, hybridizations were performed with the *NKG2F* cDNA. An *NKG2D* cDNA probe was used to identify corresponding shotgun clones. In addition, PCR probes were generated using the primer pairs D3F and D4R, D5F and D6R, Dfor and Drev (Table 1). An Expand Long Template PCR System (Boehringer Mannheim, Mannheim, Germany) using 22.5 mM MgCl<sub>2</sub> and detergents was used to assess intron sizes of *NKG2D*.

Clone inserts were sequenced using an ABI sequencer (Applied Biosystems). Sequences were assembled using the Staden package (Dear and Staden 1991), and searched against the sequence databases (Altschul et al. 1990). Further sequence comparisons were performed using the GCG package (v9.0).

#### RT-PCR and rapid amplification of cDNA ends (RACE)

RNA was prepared from the NKL cell line (Robertson et al. 1996) by acidic phenol extraction (Sambrook et al. 1989). Reverse transcription was performed using 1 µg of total RNA and random hexamers in a volume of 30 µl (Frech and Peterhans 1994; Kawasaki 1990). One µl of first-strand cDNA was used in a 10 µl RT-PCR reaction with gene-specific primers.

5' RACE reactions were performed using 100 ng poly A + mRNA purified from the total RNA using a FastTrack kit (Invitrogen, NV Leek, The Netherlands). A 5' RACE kit (Version 2.0 Gibco BRL, Eggenstein, Germany) was used with rTth DNA polymerase (Perkin-Elmer, Langen, Germany) at 60°C, and gene specific primer RDYAC11. A nested PCR was performed using the *NKG2-ex1-rev* primer and the products cloned using a pAMP kit (Gibco BRL, Eggenstein, Germany).

## Results

### Gene structure of *NKG2C* and *E* genes

The PAC clone ICRFy704D21184 (D21184) was confirmed by PCR to contain *NKG2C* and *NKG2E*, but not the *NKG2A/B* gene. *Not* I digestion and PFG analysis revealed an insert size of 90 kb. Shotgun clones were analyzed to determine the structures of the *NKG2* genes. At the genomic level, *NKG2C* and *NKG2E* were found to be highly identical (92.1% over 6661 bp) leading to the co-identification of clones from both genes in most hybridizations. Precautions were thus taken to ensure that shotgun clone sequences from the *C* gene were not mixed with those from the *E* gene: nucleotide differences in the exon sequences of the cDNAs (GenBank accession numbers X54869 and L14542) were used to assign shotgun clones to a particular gene, and overlapping shotgun clones were used in the consensus only if their sequences were 100% identical. We observed one nucleotide difference in *NKG2C* between our sequence and the database cDNA sequence: position 522 (in X54869) was reported to be an 'A', whereas our sequence identifies a 'G' residue. Hence our sequence predicts a methionine instead of an isoleucine residue in this position. Similarly we identified five T residues in the 3' UTR region of *NKG2E* at position 920 (in L14542), whereas four T residues have previously been reported. These sequence differences may represent polymorphisms.

The *C* and *E* genes give rise to predicted proteins with 22 amino acid differences (GenBank accession numbers X54869 and L14542; Adamkiewicz et al. 1994; Houchins et al. 1991). In particular the 3' ends of *NKG2C* and *E* differ substantially, since the *NKG2E* gene has an *Alu* element in its coding sequence in this region (Adamkiewicz et al. 1994). Analysis of their gene structures showed that whereas *NKG2C* has six exons, *NKG2E* was found to have seven. A splice donor site in exon VI is used in *NKG2E* which is not recognized in *NKG2C*, and just upstream of this site *NKG2C* contains three extra nucleotides. The sequences beyond the donor site of intron 6 of *NKG2E*

**Table 2** Intron-exon boundary sequences and intron phases

<i>NKG2C</i>					
Exon	Size (bp)	Intron size (bp)	Splice acceptor sequence	Splice donor sequence	Intron phase
I	187	390	–	AAGgtaaa	1
II	99	782	ctccctgcagGTT	CTTgtaag	1
III	45	544	tctattacagTCC	AAGgtaca	1
IV	152	1585	tctttattagCAC	ATGgtaag	0
V	101	877	tttcttttagAAA	TAAgtaag	2
VI	109		tcattacagGAT	–	
<i>NKG2E</i>					
Exon	Size (bp)	Intron size (bp)	Splice acceptor sequence	Splice donor sequence	Intron phase
I	187	400	–	AAGgtaaa	1
II	99	782	ctccctgcagGTT	CTTgtaag	1
III	45	547	tctattacagTCC	AAGgtaca	1
IV	155	1578	tgtttattagCAC	ATGgtaag	0
V	101	873	tttcttttagAAA	TGAgtaag	2
VI	91	3088	tcattacagGAT	ATTgtgag	0
VII	45		ttttttagAGA	–	
<i>NKG2F</i>					
Exon	Size (bp)	Intron size (bp)	Splice acceptor sequence	Splice donor sequence	Intron phase
I	187	379	–	AAGgtaaa	1
II	99	530	ctccctgcagGTT	CTTgtaag	1
III	54	540	atTTTTctagGTA	AAGgtaca	1
IV	134		ttttatttagCAC	–	
<i>NKG2D</i>					
Exon	Size (bp)	Intron size (bp)	Splice acceptor sequence	Splice donor sequence	Intron phase
IA	131	~ 22000*	–	ATGgtatg	–
IB	57	1807	cttcttttagGTA	TAGgtagg	–
II	85	1068	tcaattctagATC	CAGgtgtg	–
III	104	1761	ttttattcagGAA	GGGgtatg	1
IV	108	~ 7000*	ttttcttagAGA	ATGgtaag	1
V	93	218	tcttgacagCAT	ACTgtaag	1
VI	36	742	atTTtaataagCAT	CCGgtaag	1
VII	152	319	cctttccagAAA	CAGgttga	0
VIII	103	~ 3000*	tttcaaacagGAT	CCTgtaag	2
IX	874		ttctcttagACT	–	

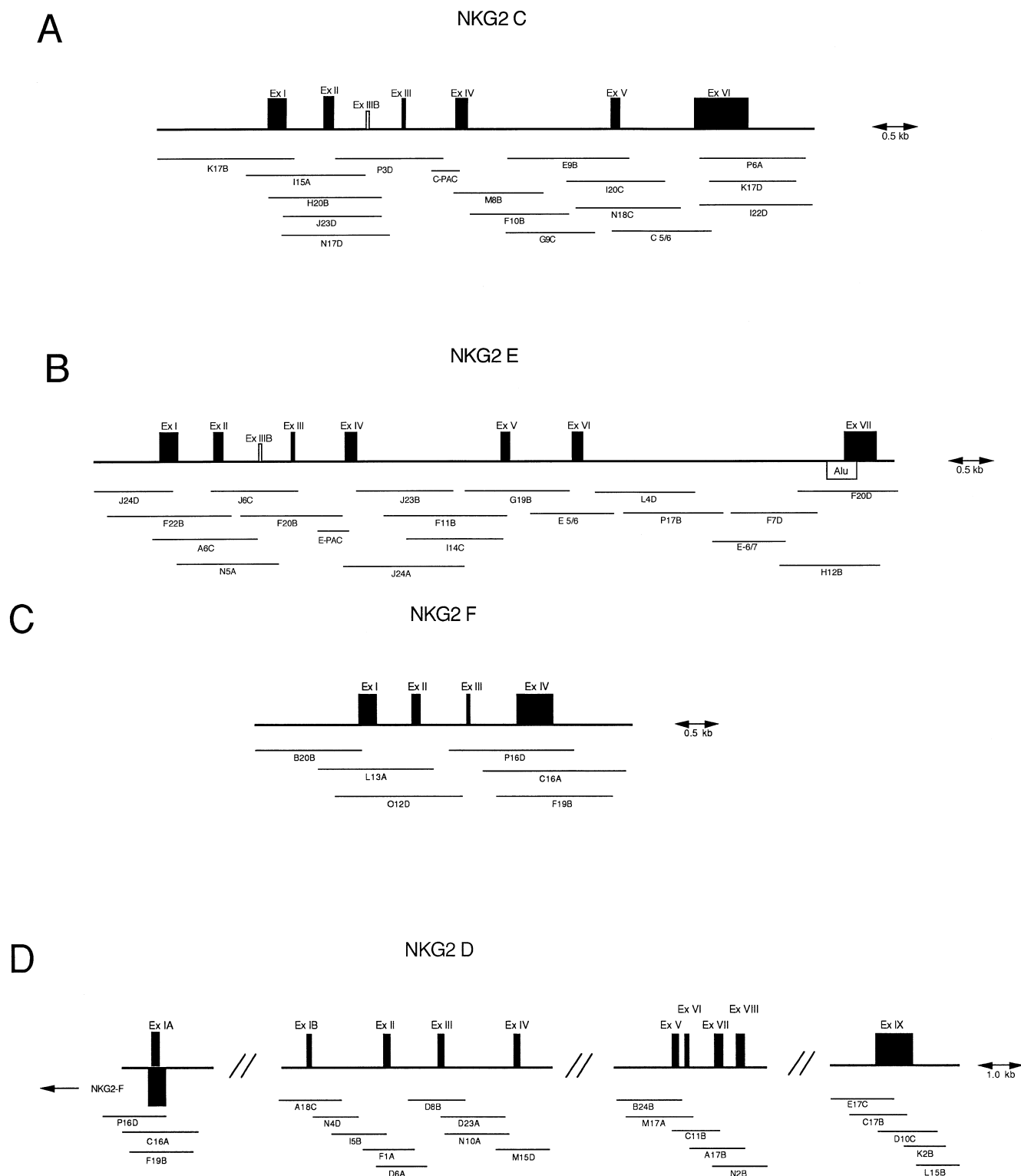
\* Intron size was estimated by PCR and Southern blotting experiments

retain a high similarity to the corresponding sequences in exon VI of *NKG2C*. Considering exons I–V of both genes, four out of the five exons are identical in size, and the patterns of codon breakage at the intron/exon boundaries are conserved (Table 2, and Sharp 1981).

An interesting feature found in intron 2 of the *NKG2C* and *E* genes is the presence of a duplicated piece of DNA (255 bp) containing a second version of exon III (termed IIIB). In each gene only the downstream version of this exon has thus far been found in cDNA sequences (GenBank accession numbers X54869 and L14542; Adamkiewicz et al. 1994; Houchins et al. 1991; Fig. 1A, B). There are 13 conserved nucleotide differences between the upstream and downstream duplicated regions (Fig. 2), which could cause the distinction between the exons in splicing. The AG/GT nucleotides in the splice acceptor and splice donor sites are however retained (Fig. 2).

### Features of *NKG2F* and *NKG2D*

Sequence analysis of shotgun clones hybridizing to *NKG2C* and *E* probes revealed a subset of clones which contained a new gene closely related to *C* and *E*. This gene has recently been published by Plougastel and Trowsdale (1997). In addition, we isolated a full-length cDNA clone, B0955 from an NK cell cDNA library. This clone contains an additional 150 nucleotides of 5' UTR sequence than has been previously reported. In parallel with Plougastel and Trowsdale (1997) we found that the gene structure of *NKG2F* differs from *C* and *E*, since it only contains four coding exons (Fig. 1). Exon IV of *NKG2F* is highly homologous to *NKG2C* and *E* genes, although two extra nucleotides (AA) are present approximately 100 bp after the beginning of exon IV (position 610 in the B0955 sequence, accession number AJ001683). This frameshift leads to a stop codon situated 20 nucleotides before the splice donor site utilized in the *C* and *E* genes. Hence, *NKG2F* is pre-



**Fig. 1A–D** Schematic representations of the gene structures of *NKG2C*, *E*, *F*, and *D*. Exons are represented as *black-filled boxes*, and shotgun clones and PCR products as *thin horizontal lines* below each gene. **A** and **B** Duplicated versions of exon III, which are present in the *NKG2C*, and *E* genes are shown as *unfilled boxes* (exon III B). Most of the sequences were obtained from shotgun clone inserts, however in five cases a PCR product was sequenced to close a small gap (C-PAC, C 5/6, E-PAC, E 5/6, E-6/7). An *Alu* sequence close to the end of *NKG2E* (Fig 1B)

crosses the intron VI – exon VII junction. **C** and **D** The *NKG2F* and *NKG2D* gene structures are shown for comparison. *NKG2F* consists of four coding exons, and hence is shorter than the other *NKG2* genes. *NKG2D* spans a larger genomic distance than the *NKG2C*, *E*, and *F* genes, and the sizes of three introns represented by ‘//’ were estimated by PCR and Southern hybridization. Some *NKG2D* transcripts have been shown to contain 131 non-coding nucleotides at their 5’ end (*exon 1 A*), which are identical to *NKG2F* exon IV sequences

**Fig. 2** The duplication of a region containing exon III in *NKG2C* and *E* genes. The region of duplication begins at nucleotide 7 in this figure and ends at position 260. Exon III (underlined version is found in *NKG2C* and *E* cDNAs) extends from nucleotide 178–213. The AG/GT nucleotides of the potential splice sites are conserved in the IIIB sequences, however residues marked in *boldface* show the differences between the two versions of the exons, which may explain splicing selectivity. *Shaded* nucleotides depict inter-gene differences between either CIIIB and EIIIB or CIII and EIII. Examination of CIIIB and EIIIB sequences shows only two nucleotide differences between them, whereas CIII and CIIIB have 16, and EIII and EIIIB have 15, 13 of which are conserved between the genes. This suggests that duplication of exon III preceded the inter-gene duplication

	1				50
CIIIB	<b>TTTTACTGTT</b>	<b>TCTTTCAAAG</b>	<b>ATCTATTACT</b>	<b>TCATTTATTT</b>	TTATAGAAAA
EIIIB	<b>TTTTACTGTT</b>	<b>TCTTTCAAAG</b>	<b>ATCTATTACT</b>	<b>TCATTTATTT</b>	TTATAGAAAA
CIII	TAAAAATGTT	TATTTCAAAG	GTCTATTACT	TTATATATTT	TTATAGAAAA
EIII	TAAAAATGTT	TATTTCAAAG	GTCTATTACT	TTATGTATTT	TTATAGAAAA
	51				100
CIIIB	AGTTAATTTT	ATTAAAGATT	<b>GTCCCCATTT</b>	TAAATAACAC	ACAAAGTTTC
EIIIB	AGTTAATTTT	ATTAAAGATT	<b>GTCCCCATTT</b>	TAAATAACAC	ACAAAGTTTC
CIII	AGTTAATTTT	ATTAAAGATT	CTCCCCATTT	TAAATAACAC	ACAAAGTTTC
EIII	AGTTAACTTT	ATTAAAGATT	CTCCCCATTT	TAAATAACAC	ACAAAGTTTC
	101				150
CIIIB	AAAGTAAGAA	ACTAAACTCA	TTATGGTTTA	<b>TCTAAATATT</b>	<b>ACTTTTTTATA</b>
EIIIB	AAAGTAAGAA	ACTAAACTCA	TTATGGTTTA	<b>TCTAAATATT</b>	<b>ACTTTTTTATA</b>
CIII	AAAGTAAGAA	ACTAAACTCG	TTATGGTTCA	TCTAGATATC	AGTTTTTATA
EIII	AAAGTAAGAA	ACTAAACTCG	TTATGGTTTA	TCTAGATATC	AGTTTTTAAA
	151				200
CIIIB	AAAATCATT	TAATTTTTCT	<b>GTTACAGTCC</b>	TGGAACAGAA	CAATTCTTCC
EIIIB	AAAATCATT	TAATTTTTCT	<b>ATTACAGTCC</b>	TGGAGCAGAA	CAATTCTTCC
CIII	AAAATCATT	TAATTTTTCT	<b>ATTACAGTCC</b>	<u>TGGAGCAGAA</u>	<u>CAATTCTTCC</u>
EIII	AAAATCATT	TAATTTTTCT	<b>ATTACAGTCC</b>	<u>TGGAGCAGAA</u>	<u>CAATTCTTCC</u>
	201				250
CIIIB	<b>CCAAATACAA</b>	<b>GAACCCAGAA</b>	<b>AAGTACATTT</b>	TTATTTTCAA	<b>AGTTCTGATA</b>
EIIIB	<b>CCAAATACAA</b>	<b>GAACCCAGAA</b>	<b>AAGTACATTT</b>	TTATTTTCAA	<b>AGTTCTGATA</b>
CIII	<u>CCGAATACAA</u>	<u>GAACGCAGAA</u>	<u>AGGTACATTT</u>	TTATTTTCAA	TGTTCTGATA
EIII	<u>CCGAATACAA</u>	<u>GAACGCAGAA</u>	<u>AGGTACATTT</u>	TTATTTTCAA	TGTTCTGATA
	251		268		
CIIIB	TTAGTACAAT	<b>TTGGAACC</b>			
EIIIB	TTAGTACAAT	<b>TTGGAACC</b>			
CIII	TTAGTACAAT	TTATATTT			
EIII	TTAGTACAAT	TTATATTT			

dicted to be 158 amino acids and has a shorter C terminal region than do *C* and *E*.

We noted several additional features of *NKG2F*. A polyadenylation signal 248 nucleotides downstream from the stop codon is used in the B0955 cDNA. In addition, this gene does not have the intra-gene duplication of exon III which is observed for *NKG2C* and *E*. *NKG2F* has 80.6% identity at the genomic level to *NKG2C* in a region of 1600 bp. Three sequence differences leading to two amino acid changes at position 29 (isoleucine/serine) and position 104 (asparagine/serine) were detected between B0955 and the genomic sequence derived from the PAC shotgun library. The third nucleotide difference which does not lead to an amino acid change occurs in the third nucleotide of the triplet coding for serine 50 which is an 'A' in the genomic sequence and a 'G' in the cDNA sequence. To investigate these differences further RT-PCR was performed using gene-specific primers, selected to generate a product containing all three variant nucleotides. Of the cloned products, two showed the same sequence as the PAC, and one clone showed the same sequence as cDNA B0955.

In parallel with the report of Plougastel and Trowsdale (1997), our analysis of the *NKG2F* sequence revealed that part of exon IV is identical to nucleotides

1–131 of the published *NKG2D* cDNA (GenBank accession number X54870). We found that *NKG2D* is situated adjacent to *NKG2F* within PAC D21184. We characterized all intron/exon boundary sequences of *NKG2D*, and sequenced the smaller introns. The larger *NKG2D* introns (intron IA, IV, and VIII) were estimated by Southern hybridization and PCR to be 22 kb, 7 kb and 3 kb respectively (Table 2, Fig. 4). While the originally described transcript for *NKG2D* contained 131 bp of *NKG2F* exon IV (*NKG2D* exon IA) at the 5' end, additional cDNAs for *NKG2D* have been isolated and reported to start in exon IB, hence not encompassing any *NKG2F* sequences (Houchins et al. 1991). For this reason we have referred to the *NKG2D*-specific exons as exon IB to IX, and to the exon derived from the *NKG2F* sequence, which may precede exon IB as *NKG2D* exon IA (Fig. 1D).

#### Characterization of *NKG2* cDNA 5' end sequences

*NKG2A/B* contains one separate 5' UTR exon upstream of the first coding exon (Plougastel et al. 1996). However *NKG2F* has 182 bp upstream of the start ATG, which are not contained in a separate 5' non-coding exon. We compared *NKG2E* and *C* cDNA se-

quences (accession numbers X54869 and L14542) to the newly derived genomic sequence, and found no evidence for separate upstream 5' UTR exons. We performed 5' RACE for *NKG2C* to test whether the published cDNA sequence (X54869, Houchins et al. 1991) was incomplete at the 5' end. This seemed a strong possibility for this gene since only 7 bp upstream of the start methionine are present in the database sequence (Houchins et al. 1991) compared with 45 bp for *NKG2E*. Furthermore, the genomic sequences of *NKG2C* and *E* show a high identity to the 182 bp of 5' UTR sequence of *NKG2F*. A RACE product was generated which extended the *NKG2C* cDNA sequence at the 5' end by 38 bp. Hence *NKG2C* appears to end at the same position as *NKG2E*, and no further 5' UTR exons were identified.

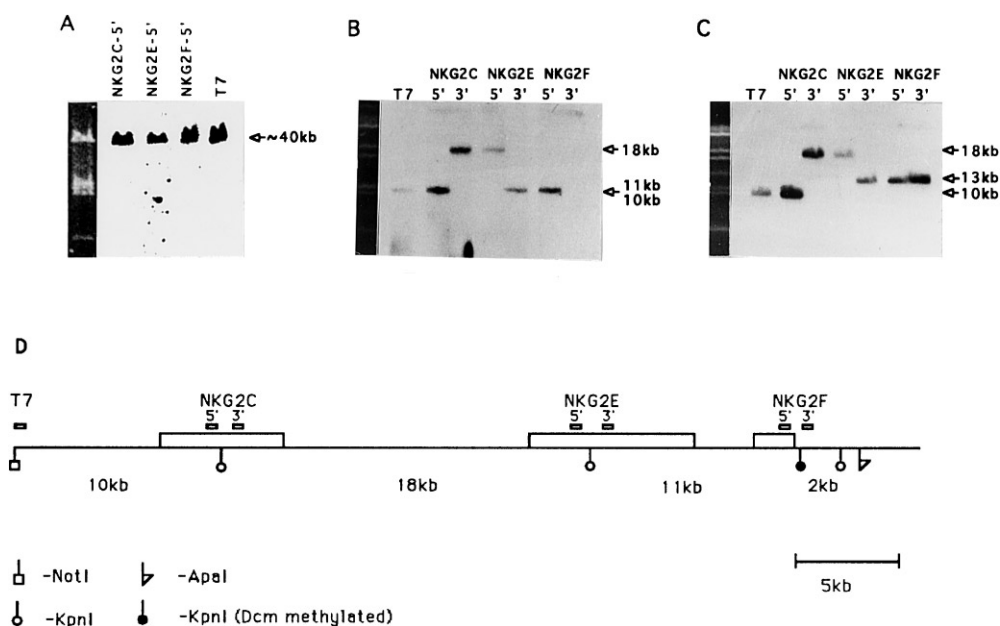
#### Linkage and orientation of the *NKG2C*, *E*, *F*, and *D* genes

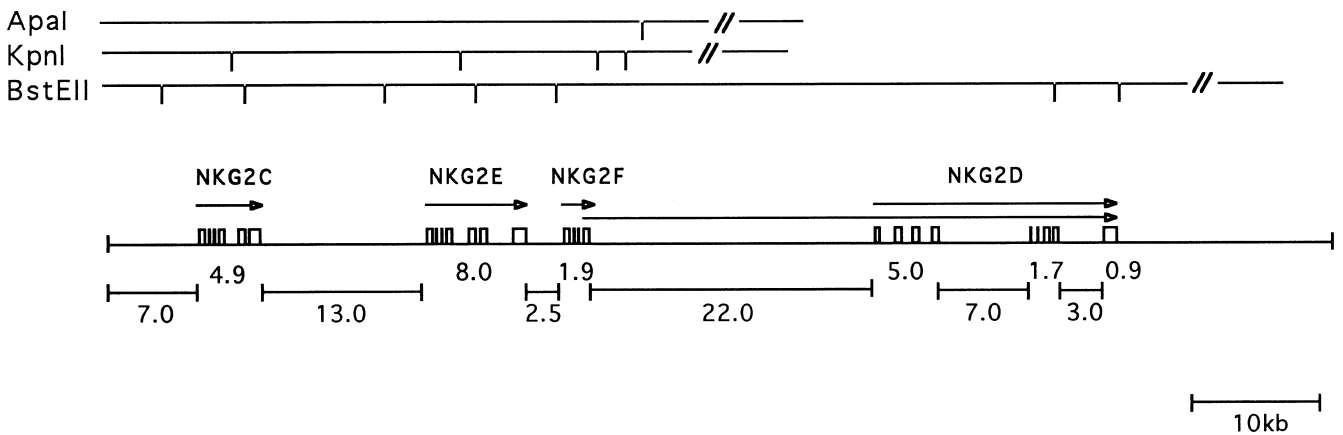
The PAC D21184 was characterized further to establish the genomic organization of the *NKG2C*, *E*, *F*, and *D* genes. By restriction enzyme mapping it was possible to establish that *NKG2C*, *E*, and *F* are situated on a 40 kb *Not* I/*Apa* I fragment on the T7 side of the PAC insert (Fig. 3A). Further hybridizations to *Not* I and either *Kpn* I (Fig. 3B) or *Asp* 718 (Fig. 3C) digests revealed the gene order of T7-*NKG2C*-*NKG2E*-*NKG2F*-SP6 with respect to the PAC ends (Fig. 3D). Restriction sites for the isoschizomers *Kpn* I / *Asp* 718 were found in intron IV of the established gene sequences of *NKG2C*, *NKG2E*, and the corresponding region of *NKG2F*. An additional site is located 2.5 kb downstream of *NKG2F*. The *Kpn* I recognition sequence in *NKG2F* overlaps with a dcm methylation site and is therefore not cut by the isoschizomer *Asp* 718. Gene

specific oligonucleotides designed to hybridize close to the ends of the restriction fragments were used to unequivocally establish the relative distance and orientation of the *NKG2* family members as shown in Fig. 3B, C. While *Kpn* I digestion established the order and identical orientation of *NKG2C* preceding *NKG2E* and *NKG2F*, *Asp* 718 analysis improved the separation of the restriction fragments confirming the results. The 5' end of *NKG2C* was found to be located approximately 6.5 kb from the T7 end of the PAC cloning site, the distance between *NKG2C* and *NKG2E* was estimated to be 13 kb, and between *NKG2E* and *NKG2F* approximately 2.6 kb (Fig. 3D).

*NKG2D* is present on a 40 kb *Bst*EII fragment which also includes *NKG2F* (data not shown). A primer specific for the start of *NKG2D* intron VIII hybridized to the identical *Bst* EII fragment, whereas a probe from the end of intron VIII detects a 5 kb *Bst* EII band. Hence the 5' end of *NKG2D* is adjacent to *NKG2F* and this gene shows the same transcriptional orientation as *NKG2C*, *E*, and *F* (Fig. 4). This result confirms the genomic sequence analysis, and suggests that inter-gene splicing produces the *NKG2D* alternative splice product. Hybridization with an oligonucleotide specific for

**Fig. 3** Linkage and orientation of *NKG2C*, *E*, and *F*. PAC D21184 was digested by *Not*I / *Apa* I (A), *Not* I/*Kpn* I (B), and *Not* I / *Asp* 718 (C). *Asp* 718 is an isoschizomer of *Kpn* I sensitive to dcm methylated sites. The resulting fragments were separated by PFG or regular agarose gel electrophoresis, stained by ethidium bromide (first lane of A, B, C) and blotted onto nylon filters. The blots were then probed with radioactively labeled gene-specific oligonucleotides detecting sequences 5' and 3' of the *Kpn* I restriction sites identified for each gene. An oligonucleotide hybridising to the T7 promoter sequence of the PAC vector was used to link the *NKG2C* gene to the cloning site. A schematic drawing of the PAC restriction fragments as detected by the specific oligonucleotides is shown in D





**Fig. 4** Map of the genomic region containing *NKG2C*, *E*, *F*, and *D*. The data obtained for the linkage and orientation of the four *NKG2* family members analyzed were combined to generate a map of the respective gene locus. The exons are indicated by boxes, the transcribed regions corresponding to the cDNA sequences are represented by arrows. The sizes of the genes were calculated from the established sequences and are given in kb below the individual genes. Intergenic distances were estimated from Southern blot analysis of the respective restriction fragments. The size estimates of introns IV and VIII of *NKG2D* were based on PCR products (Dfor and Drev, *NKG2D*-in8F, and R)

exon IV of *NKG2F* did not detect more than one copy of *NKG2F* in PAC D21184 (data not shown).

#### Comparison of amino acid sequences

We performed a sequence comparison of several C-type lectin receptors localized in 12p13. A multiple sequence alignment was performed and the data are represented in a dendrogram (Fig. 5), where the clustering of the receptors reflects the similarities of their amino acid sequences. In fitting with the genomic sequence analysis, *NKG2E* and *C* are shown to be most similar, and *NKG2F* is more similar to these two genes than to *NKG2A*. *NKG2D* is no more similar to the *NKG2A*, *C*, *E*, and *F* genes than is *CD94*.

#### Discussion

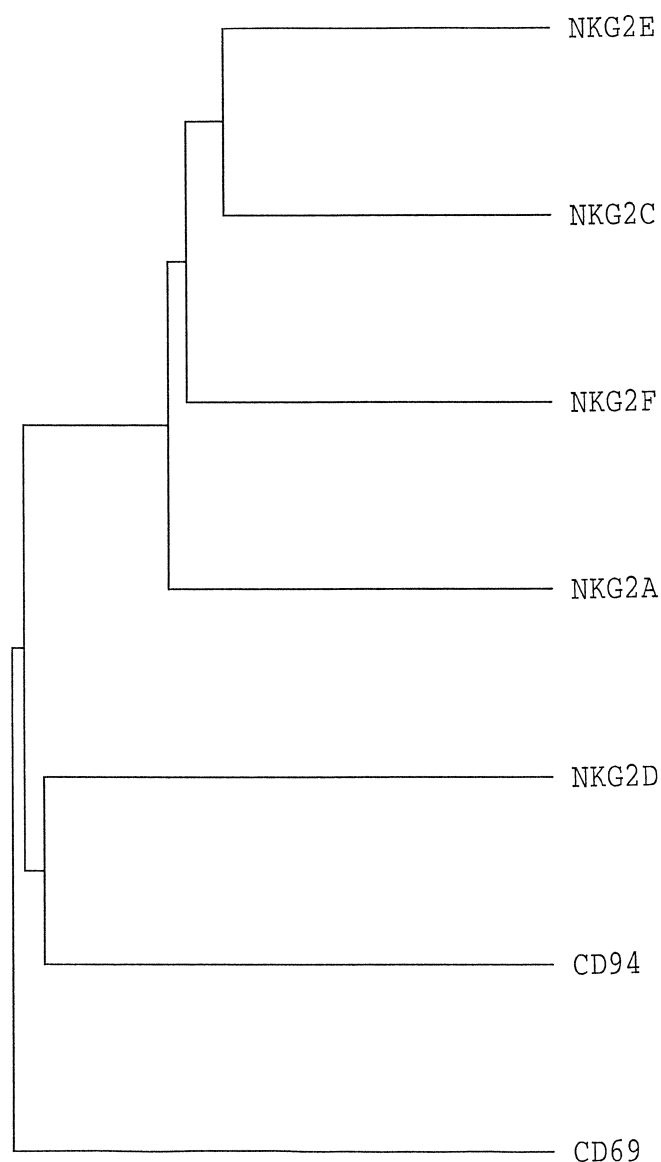
We used a shotgun sequencing approach to establish the gene structures of *NKG2C* and *E*. The newly isolated *NKG2F* gene was also examined in detail at both the cDNA and genomic DNA level. The *NKG2C*, *E*, and *F* genes were found to be closely related at the genomic level, and localized adjacent to *NKG2D*. We showed that the four genes are clustered together within one 90 kb PAC clone, and each has the same transcriptional orientation.

Several insights into the evolution of this gene family were gained by examination of the genomic sequences of the *NKG2* genes. *NKG2C* and *E* contain a duplicated version of exon III, which has not yet been

identified in cDNA sequences despite the conservation of the AG/GT splice sites. This version of exon III may lie in a less favorable genomic context which does not allow splicing to occur, or splicing might occur in certain circumstances. Exon IIIB in *NKG2C* and *E* differs from the transcribed exon (Houchins et al. 1991) by 4 and 3 nucleotides, respectively. One of these differences would result in an amino acid change if these exons were to be utilized. Exon III codes for the 'stem' of the receptor and the corresponding region of mouse Ly49 receptors has been shown to contribute to MHC class I specificity (Brennan et al. 1996). This duplicated exon could thus contribute to ligand specificity. Both versions of exon III in *NKG2C* are highly homologous to their counterparts in *NKG2E*. This similarity is higher than the similarity of duplicated regions within either gene, which suggests that the duplication of exon III preceded the inter-gene duplication.

*NKG2E* differs from *NKG2C* by the presence of *Alu* sequences close to the 3' end of the gene. This phenomenon has been suggested to represent an evolutionary mechanism of introducing variability within protein domains (Makalowski et al. 1994). Since the 3' region codes for the extracellular domain of the *NKG2* receptors, it is feasible to imagine that such variability may directly affect ligand specificity. A splice acceptor site within the repeat is used such that the last 17 amino acids of *NKG2E* are derived from the *Alu* sequences. Recently an alternative splice product of *NKG2E* has been identified which does not contain exon VII: in this case the splice donor site of exon VI in *NKG2E* is not used and therefore the C terminal end is very similar to *NKG2C* (T. Bellon, and M. Lopez-Botet, personal communication).

*NKG2F* is truncated at its 3' end compared to other *NKG2* genes and it is not yet known if this gene encodes a functional receptor. Two versions of *NKG2F* were isolated exhibiting 2 amino acid differences. One of these, Ser to Ile, may significantly change the function of the protein. The nucleotide differences may represent linked allelic polymorphisms, with only two variants found in several DNA/RNA sources. Alternatively, these transcripts could represent highly similar genes.



**Fig. 5** A dendrogram showing the clustering relationships between several C-type lectin receptors which map to 12p13. A 'Pileup' multiple sequence alignment (GCG) was performed using the predicted amino acid sequences. The relationships between the receptor molecules reflect the similarity of their sequences. NKG2C is very similar to NKG2E as was revealed by comparison of their genomic sequences. In addition, NKG2F and NKG2A are very similar to NKG2C and E. NKG2D is no more similar to the NKG2 family than to CD94

The published *NKG2D* cDNA contains 5' sequences identical to exon IV of *NKG2F*. Our data shows that this 5' exon is indeed derived from the *NKG2F* gene. Plougastel and Trowsdale (1997) were able to perform RT-PCR between *NKG2F* and *NKG2D*, although the expected gene product would be the same as that derived from the *NKG2F*-specific mRNA. However, detection of *NKG2D* transcripts that do not contain any *NKG2F* sequences (Houchins et al. 1991) suggests that there may also be a functional *NKG2D*-specific pro-

moter. Interestingly, the splice donor site used in *NKG2C* and *E* to splice exon IV is used in the inter-gene splicing of *NKG2F* and *D*. This splice donor site is apparently overlooked in *NKG2F* transcripts such as that contained in the B0955 cDNA.

*NKG2D* is only remotely related to the other family members (Houchins et al. 1991; Hofer et al. 1992). It is also the only *NKG2* gene thus far found in rodents (Dissen et al. 1996), which also have *CD69* and *CD94* homologues (Dissen et al. 1997). This indicates these genes existed before the divergence of the rodent and human genomes. Comparisons using the human *NKG2*, *CD94*, and *CD69* peptide sequences suggests that *CD94* and *NKG2D* are equally distant to the *NKG2* family. Indeed, *NKG2D* may be more closely related to *CD94* than it is to the other *NKG2* receptors. Since *CD94* dimerises to the variant *NKG2* receptors, it will be interesting to know the function if any, of *NKG2D*.

Both the C-type lectin and Ig-like receptors are involved in the regulation of NK cytotoxic function. Although this process is not well understood, a conserved amino acid sequence has been recognized in the cytoplasmic N terminal part of some Ig-like receptors and *NKG2A*. This sequence is termed an immunoreceptor tyrosine-based inhibitory motif (ITIM). *NKG2C*, *E*, and *F* do not contain an ITIM, but contain either a lysine or an arginine residue in their transmembrane domains. These negatively charged residues are believed to allow interactions with other transmembrane signal transducing molecules (Lanier et al. 1998) in the process of NK cell activation.

The genomic organization of the *NKG2* genes indicates that this family has arisen by duplication from a single ancestral gene, and a variety of mechanisms (such as alternative splicing and the use of *Alu* repeats in the coding sequence) have been used to generate diversity. Such diversity is likely to have been essential in order to follow the evolution of NK receptor ligands, for example MHC molecules. Further work is required to fully identify the different types of ligands for all members of the *NKG2* family, in order to completely evaluate the function and molecular evolution of this gene cluster.

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