The autocrine motility factor receptor gene encodes a novel type of seven transmembrane protein[1]

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Abstract Autocrine motility factor receptor (AMFR) is a cell surface glycoprotein of molecular weight 78 000 (gp78), mediating cell motility signaling in vitro and metastasis in vivo. Here, we cloned the full-length cDNAs for both human and mouse AMFR genes. Both genes encode a protein of 643 amino acids containing a seven transmembrane domain, a RING-H2 motif and a leucine zipper motif and showed a 94.7% amino acid sequence identity to each other. Analysis of the amino acid sequence of AMFR with protein databases revealed no significant homology with all known seven transmembrane proteins, but a significant structural similarity to a hypothetical protein of *Caenorhabditis elegans*, F26E4.11. Thus, *AMFR* is a highly conserved gene which encodes a novel type of seven transmembrane protein.

Key words: Autocrine motility factor receptor; RING-H2; Leucine zipper; Seven transmembrane; Expressed sequence tag

1. Introduction

Autocrine motility factor (AMF) is a tumor secreted molecular weight 55 000 cytokine that regulates cell motility in vitro as well as invasion and metastasis in vivo [1]. Recent studies have demonstrated that mouse and human AMFs are identical to the products of previously cloned genes, neuroleukin (NLK) and phosphohexose isomerase (PHI). PHI catalyzes isomerization of glucose 6-phosphate to fructose 6-phosphate and is specific for both sugars. Mouse AMF exhibits the enzymatic properties of PHI and rabbit heart PHI stimulates the motility of mouse fibrosarcoma cells similarly to mouse AMF [2]. Recently, maturation factor (MF), which mediates the differentiation of human myeloid leukemic cells to terminal monocytic cells, was also found to be identical to PHI [3]. Thus, AMF, NLK, PHI and MF are single gene products with pleotropic functions [4].

AMF stimulates random and directed cell motility via its receptor. AMF receptor (AMFR) was identified as a 78 kDa cell surface glycoprotein (gp78) and a monoclonal antibody against it stimulates cell motility in vitro and the metastatic ability in vivo by mimicking the effect of the AMF ligand [5,6]. Moreover, it has been shown that increased expression of AMFR correlates with a high incidence of recurrence and a shortened survival in patients with bladder cancer, colorectal cancer, oesophageal cancer and gastric cancer [7–10].

To elucidate the functional role of AMFR in cancer invasion and metastasis, it is pertinent to isolate a mouse homologue of the human *AMFR* gene to construct a mouse model defective in the *Amfr* gene. We therefore cloned the full-length cDNA for the mouse *Amfr* gene. In the process of this cloning, we uncovered that the human AMFR cDNA reported previously [11] was only the 3′-partial sequence of this gene. Thus, we further isolated the full-length cDNA for the human *AMFR* gene. Full-length cDNAs for the human and mouse *AMFR* genes encode a seven transmembrane protein with a RING-H2 motif and a leucine zipper motif and showed a 94.7% amino acid sequence identity with each other. Protein sequence database analysis revealed that the AMFR protein had no significant homology with registered protein sequences including the ones of seven transmembrane protein, but a significant structural similarity to a *Caenorhabditis elegans* hypothetical protein, F26E4.11. Thus, the *AMFR* gene is an evolutionarily conserved gene which encodes a novel type of seven transmembrane protein having the RING-H2 and leucine zipper motifs.

2. Materials and methods

2.1. Screening and isolation of cDNA clones

A mouse expressed sequence tag (EST) clone (GenBank accession number AA260491) was amplified from mouse lung cDNA by using a primer pair of 5′-TAATATTGCTGATGGCAGTC-3′ and 5′-TCTAGGCGAGGACTGAGGTC-3′. A partial cDNA fragment of the human *AMFR* gene was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from mRNA of a DLD-1 colon cancer cell line using a primer pair of 5′-GATGTGGCCCAGTACCTGCTCTCA-3′ and 5′-ATTTGCAGTGTTTCTAAGGGGA-3′. The sequences of the primers were designed from those of two EST clones (GenBank accession numbers M62018 and AA479243), which showed homologies with a mouse Amfr cDNA clone (GenBank accession number AF124144) (Fig. 1). The PCR conditions used were as follows: 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 2 min. cDNA libraries from mouse lung, mouse testis and a HeLa cell line were used to isolate full-length cDNA clones. Isolated clones were sequenced by the A.L.F. DNAsequencer II with the AutoRead Sequencing kit (Amersham Pharmacia Biotech, Upsala, Sweden).

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[1] The nucleotide sequence data reported in the paper will appear in the GenBank/EMBL/DDBJ database with the accession numbers AF124144 and AF124145.

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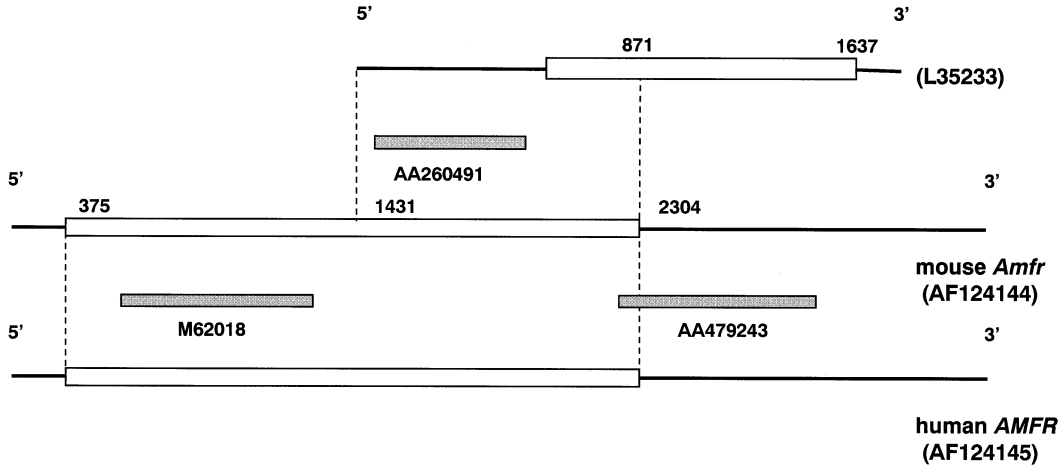


Fig. 1. cDNA structures for the mouse and human *AMFR* genes. Open boxes indicate a region of the ORFs of the *AMFR* genes. Filled boxes indicate a mouse EST clone (GenBank accession number AA260491) and two human EST clones (GenBank accession number M62018 and AA479243).

2.2. Homology search and analyses of nucleotide and amino acid sequences

Sequence homologies of nucleotides and predicted amino acids were analyzed through the GenomNet WWW server using BLASTN, BLASTX and BLASTP searches of the non-redundant database [12]. Predictions of transmembrane regions were performed through the GenomNet WWW server using the SOSUI program.

2.3. Northern blot analysis

Mouse multiple tissue Northern (MTN) blot, containing 2 μg of poly (A)+ RNA from a variety of organs, was obtained from Clontech (Palo Alto, CA, USA).

Northern blot hybridization was performed under the stringent conditions as described previously [13]. Filter was hybridized with a DNA probe corresponding to bases 1120–2333 of the cloned *Amfr* cDNA fragment.

2.4. Chromosome mapping of the mouse Amfr gene

An interspecific backcross panel generated from (C3H/HeJ × *Mus spretus*) F1 × C3H/HeJ was used for mapping of the mouse *Amfr* gene locus [14]. Genotypes of Amfr for 134 individuals in this panel were determined by a restriction fragment length polymorphism (RFLP) observed in the PCR product of 740 bp in size that was amplified from the 3′-non-coding region of the gene. An RFLP between the two mouse strains SEG (*M. spretus*) and C3H/HeJ was identified by using the *Xba*I enzyme. The sequences of the primers were 5′-GACATTTCTCTTCCCCTTAG-3′ and 5′-TAATGAGACCCACAGGAACA-3′, corresponding to nucleotides 2465–2485 and 3185–3205, respectively. Mapping was carried out by analyzing the segregation of these variants relative to known markers. The data generated in this study were analyzed by Map Manager Version 2.5.6 [15].

3. Results

3.1. Isolation and characterization of the human and mouse AMFR genes

We searched for mouse ESTs homologous to the human *AMFR* gene in the GenBank EST database. A mouse EST clone (GenBank accession number AA260491) was identified as having an 86% sequence homology with a cDNA clone of the human *AMFR* gene (GenBank accession number L35233) [16]. Thus, this EST was amplified by PCR from mouse lung cDNA and used as a probe for the screening of mouse cDNA libraries. Two independent clones, ML4 and MT68, were isolated from mouse lung and testis cDNA libraries, respectively. Sequence analysis revealed that ML4 and MT68 overlapped and contained the entire sequence of AA260491. A contiguous sequence of 3.5 kb was obtained from these clones and was verified by RT-PCR and sequencing (GenBank Accession number AF124144). The nucleotide sequence of AF124144 revealed 76% identity with that of L35233 and contained an open reading frame (ORF) of 643 amino acids. However, the ORF of the mouse cDNA clone, A124144, was markedly different from that of L35233. The 3′-end of the ORF in A124144 was partly matched to the 5′-end of the ORF in L35233 (Fig. 1). Therefore, it was assumed that L35233 is a partial sequence of the human AMFR cDNA. To explore this, a cDNA clone of 3 kb insert, H7, was isolated from a human HeLa cDNA library (GenBank accession number AF124145) using a cDNA fragment of the human *AMFR* gene as a probe. The H7 clone showed 88.3% identity with the nucleotide sequence of the mouse cDNA and contained an ORF of 643 amino acids showing a significant homology (94.7% identity) with the mouse predicted amino acid sequence. Comparison of the nucleotide sequence of the H7 clone, AF124145, with that of the previously isolated clone, L35233, revealed that there were four one base deletions and two one base insertions in L35233, resulting in the frame-shift of the ORF. Therefore, we concluded that the cDNA clones isolated here are the full-length ones for both the mouse *Amfr* and the human *AMFR* genes.

Hydrophobic analysis of the amino acid sequence encoded by the human *AMFR* gene revealed a putative seven transmembrane domain topology (Fig. 2B and C). Sequence analysis also revealed the presence of a RING-H2 motif, which is a zinc finger variant [17], and a leucine zipper motif [18]. Fig. 2D shows an alignment of the RING-H2 motif in AMFR and several other RING-H2 containing proteins. Furthermore, AMFR protein included a potential *N*-glycosylation site and several potential O-glycosylation sites (Fig. 2A and B), supporting the previous finding that gp78 is glycosylated with both N- and O-glycosaccharides [19]. A putative seven transmembrane domain, RING-H2 motif, leucine zipper motif and a potential *N*-glycosylation site were all conserved in the mouse AMFR proteins (Fig. 2A and B).

3.2. Homology of the AMFR with C. elegans protein F26E4.11

Comparison of the protein encoded by the *AMFR* gene with protein sequence databases showed no significant homol-

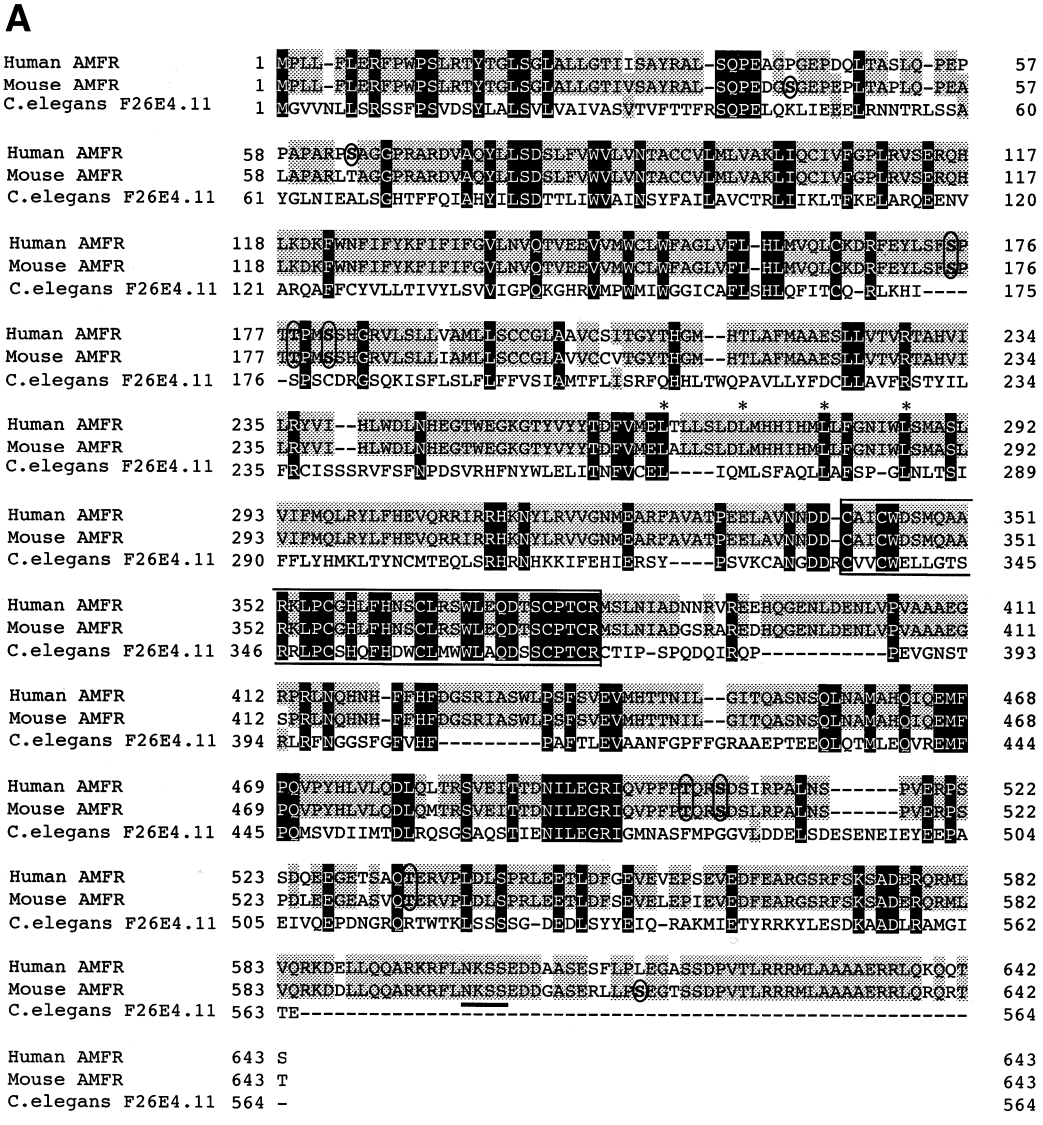
ogy with any known proteins except a *C. elegans* hypothetical protein F26E4.11 (GenBank accession number Z81070), that was predicted from a computer analysis of contiguous nucleotide sequences from chromosome I [20]. The amino acid sequence of F26E4.11 showed 23% identity and 43% similarity with that of human AMFR protein (Fig. 2A). The hydropathy profile of F26E4.11 was extremely similar to those of the human and mouse AMFR proteins and the RING-H2 motif was also conserved in the F26E4.11 sequence (Fig. 2A, C and D). This result indicates that F26E4.11 is a *C. elegans* homologue of the human *AMFR* gene.

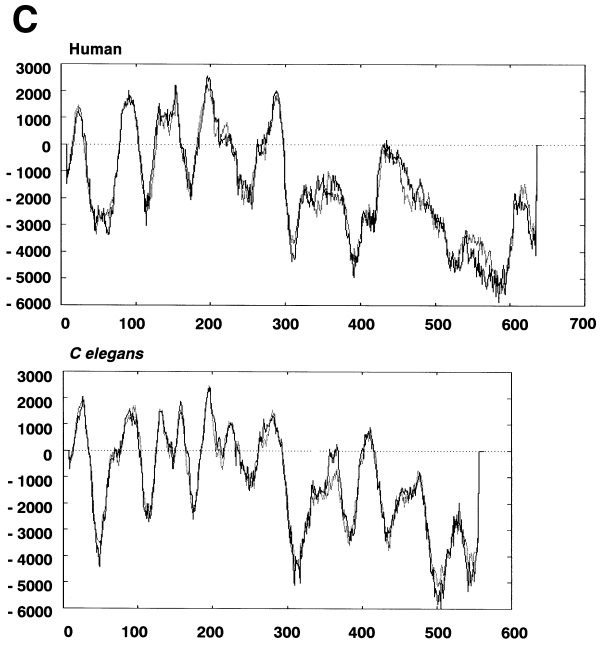
3.3. Expression of the Amfr gene

To determine the distribution of the *Amfr* gene expression in normal tissues, we performed multiple tissue Northern blot analysis of the mouse *Amfr* gene. A mRNA transcript of 3.5 kb was detected in the heart, brain, lung, liver, skeletal muscle, kidney and testis. No *Amfr* mRNA transcript was detected in the spleen (Fig. 3).

3.4. Chromosome mapping of the mouse Amfr gene

The human *AMFR* gene has been mapped to chromosome 16q21 [21]. To assign the chromosomal location of the mouse





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AMFR	Human	341	CAIC---WDSMQAARK---LFCGHLFHNSCLRSW------LEQD--T-SCFTC	378
AMFR	Mouse	341	CAIC---WDSMQAARK---LFCGHLFHNSCLRSW------LEQD--T-SCFTC	378
F26E4.11	C.elegans	338	CVVC---WELLGTSRR---LFCSHQFHDWCLMWW------LAQD--S-SCFTC	372
Goliath	Drosophila	126	CAICIEAYKPTDTIRI---LFCKHEFHKNCIDPW------LIEH--R-TCPMC	166
Prajal	Mouse	347	CPICCSEYVKGEVATE---LFCHHYFHKPCVSIW------LQKS--G-TCPVC	387
Neurodap1	Rat	633	CPICCSEYIKDDIATE---LFCHHFFHKPCVSIW------LQKS--G-TCPVC	673
TPRD	Human	1954	CEICHEVFKSKNV-RV---LKCGHKYHKGCFKQW------LKGQ--S-ACPAC	1996
Rapsyn	Human	363	CALCGESIGEKNSRLQA--LFCSHIFHLRC----------LQNNGTR-SCPNC	402
Rapsyn	Mouse	363	CGLCGESIGERNSRLQA--LFCSHIFHLRC----------LQNNGTR-SCPNC	402
Rapsyn	Torpedo	363	CGLCGESIGDQNSQLQA--LFCSHLFHLKC----------LQTNGNR-GCPNC	402
RING-H2 finger			C C C H H C C C	
			1 2 3 1 2 4 5 6	

Fig. 2. (A) Alignments of human AMFR, mouse AMFR and *C. elegans* F26E4.11. Black and gray boxes indicate identical and conservative amino acids. The leucine zipper motif is denoted by asterisks. The RING-H2 motif is boxed, a N-glycosylation site is underlined and O-glycosylation sites are enclosed by circles. (B) Linear arrangement of the human AMFR protein. The scale above provides amino acid numbers. The hatched boxes indicate regions of putative transmembrane domains. The position of the RING-H2 motif is shown by a more densely hatched box. The ▼ symbols refer to O-glycosylation sites. (C) Hydropathy profiles of human AMFR and *C. elegans* F26E4.11. The plot was generated by the method of Kyte and Doolittle [35]. The ordinate indicates the hydropathy index and hydrophobic and hydrophilic values are plotted above and below the central line, respectively. The abscissa shows the amino acid numbers. (D) Alignment of the RING-H2 motif of AMFR with those of several other proteins found in a BLAST search of the SwissProt plus PIR database [11]. Black and gray boxes indicate identical and conservative amino acids. The number (1–6 and 1–2) below each of the conserved Cys and His refers to the potential metal binding ligands C1–C6 and H1–H2, respectively.

Amfr gene, interspecific backcross analysis was performed [14]. The *Amfr* locus was mapped to chromosome 8 and tightly linked to the *D8Mit11* locus (Fig. 4A and B). This region is known to be syntenic with human chromosome 16q21.

4. Discussion

In this study, we cloned the full-length cDNAs for both human and mouse *AMFR* genes. Structural analysis revealed that the AMFR protein has a putative seven transmembrane domain with a RING-H2 motif and a leucine zipper motif. High levels of sequence homology between human and mouse AMFR proteins as well as the presence of a structurally similar sequence in *C. elegans* indicate that this gene is highly conserved among different species. Expression of this gene in diverse organs suggests that its expression plays an important and/or common role in the maintenance of various types of cells in different organs.

Most proteins with a seven transmembrane domain are known as members of guanine nucleotide binding protein (G-protein)-coupled receptors [22]. This result lends credence to the previous findings showing that AMF interacts with a cell surface receptor coupled with a pertussis toxin-sensitive G-protein to initiate cell motility [23]. Interestingly, AMFR protein possessed a RING-H2 motif and a leucine zipper motif. The RING-H2 motif is structurally similar to the RING finger motif, but the fourth cysteine in the RING finger motif is replaced by histidine in the RING-H2 motif [17]. Although many RING finger proteins are reported to have DNA binding activity as in the case of zinc finger proteins, it has been suggested that most of the RING-H2 finger proteins do not bind DNA [17]. In particular, a subfamily of the RING-H2 finger proteins is known to be associated with the membrane.

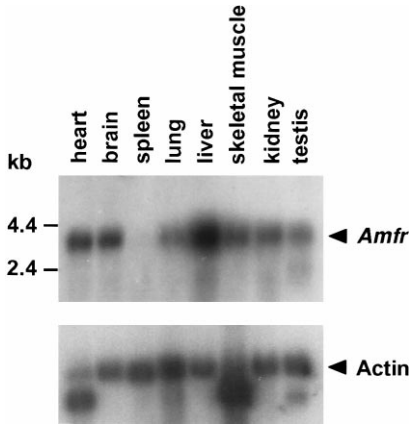

Fig. 3. (A) Northern blot analysis of the *Amfr* gene in various mouse tissues. Mouse MTN Blot (Clontech) was hybridized with an Amfr cDNA probe. A mRNA transcript of 3.5 kb is indicated by an arrowhead. The membrane was re-hybridized with a human β-actin probe.

For instance, rapsyn has been implicated in the clustering and aggregation of acetylcholine receptors and is found to be associated with the plasma membrane [24]. Neurodap1 is associated with the cytosolic face of the membrane of the endoplasmic reticulum and Golgi apparatus and with the postsynaptic region on the cytoplasmic membrane [25]. Recent studies demonstrated that a RING-H2 motif in the yeast Ste5 protein, which is a scaffold for the mitogen-activated protein kinase cascade components in a yeast pheromone response pathway, is not only required for binding to Ste4 but also required (directly or indirectly) for Ste5 oligomerization [26]. Based on these observations, it has been suggested that the RING-H2 motif is involved in a protein-protein or a protein-lipid interaction. On the other hand, the leucine zipper motif contains four leucyl residues repeated in every seventh amino acid and mediates protein dimerization [27]. rZIP is a multi-domain protein which regulates cell fate determination during *Dictyostelium* development [28]. As with AMFR, rZIP contains a RING finger motif and a leucine zipper motif and both the RING finger motif and the leucine zipper motif contribute to homodimer formation of rZIP [28]. These results suggest that the leucine zipper motif of the AMFR protein is involved in its homodimer formation together with the RING-H2 motif and AMFR proteins act as a homodimer.

The identification of AMFR as a seven transmembrane domain receptor raised the question of whether AMF belongs to the C-C, C-X-C gene family of chemoattractants, such as macrophage inflammatory protein-1a/RANTES and monocyte chemoattractant protein-1, and AMFR to the CXC-receptor (CXCR) gene family [29,30], since the vast majority of CXCR proteins signal via heterotrimeric G-proteins. A homology search has revealed that AMF does not contain the C-C or C-X-C motif and AMFR is not homologous with the CXCR gene family. Thus, it was concluded that the C-C, C-X-C chemokines and their receptors are different from the AMF/AMFR. However, AMF contains a similar motif, i.e. C-X-X-C (Cys_{330}-Phe-Glu-Cys_{333}). A GenBank search revealed that this motif is very rare and is present only in the disulfide isomerase protein family as defined as the thioredoxin-box motif [31] and in one cytokine known as the macrophage migration inhibitor factor (MIF) [32,33]. AMF and MIF share several structural and functional properties. Both may be secreted by activated T-cells by a non-classical pathway, both lack a signal peptide and both form an intramolecular disulfide bridge [4,32,33]. Since the MIF disulfide bridge was shown to be in the motif where its integrity is essential for function [32] and since reducing agents inhibit the AMF function, we question whether this C-X-X-C motif regulates the structural integrity and function of AMF. The comparative analysis of AMF and MIF is incomplete due to the fact that the MIF receptor has not yet been identified. The results presented here lay the foundation for future research on the molecular and evolutionary significance of the AMF/AMFR system in the further understanding of the motile signaling pathway.

Expression of AMFR relates to cell motility-regulating effects and also may play an important role in tumor cell invasion and metastasis [34]. Here, we cloned the full-length cDNAs for both human and mouse *AMFR* genes. The availability of the cDNA clone containing the complete coding region for both the human and mouse *AMFR* gene will now permit detailed studies to elucidate the functional role of AMFR in cancer invasion and metastasis.

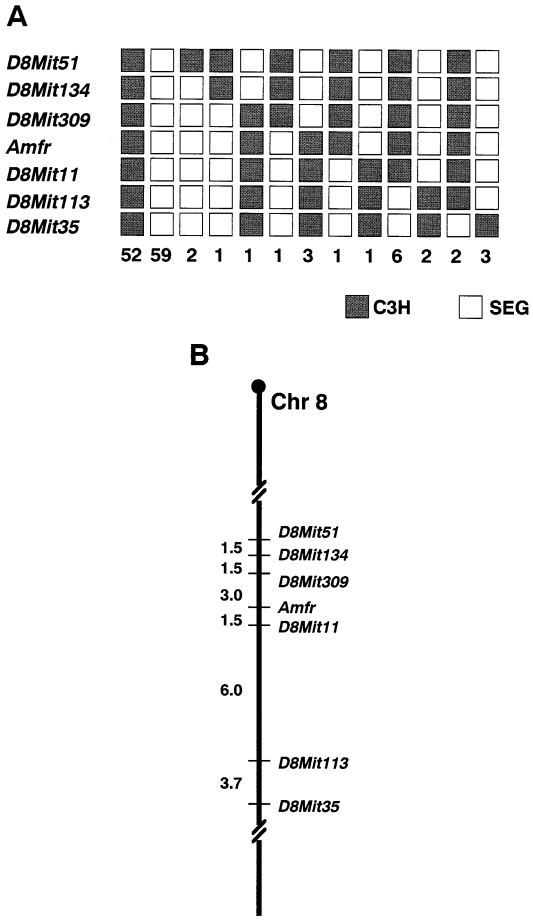

Fig. 4. (A) Haplotype data of 134 progenies of (C3H/HeJ×SEG) F1×C3H/HeJ for the loci flanking the Amfr locus. The microsatellite and Amfr loci are listed at the left. Each column represents a chromosomal haplotype identified in the progenies. Filled box, C3H/HeJ allele; open box, SEG allele. The number of progenies for each haplotype is listed at the bottom of each column. (B) A genetic map around the Amfr locus constructed from the haplotype data. Recombination frequencies expressed as genetic distances in cMorgans are shown on the left.

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