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Novel Serine Proteases Encoded by Two Cytotoxic T Lymphocyte–Specific Genes

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Genes that are expressed exclusively in cytotoxic T cells should encode proteins that are essential for target cell lysis in cell-mediated immune responses. The sequences of two cytotoxic T lymphocyte-specific complementary DNA's (cDNA's) suggest that the two genes encode serine proteases. A full-length cDNA corresponding to one of the genes was isolated and sequenced. The predicted protein resembles serine proteases in that it includes all the residues that form the catalytic triad of the active site of serine proteases. Moreover, it has sequence characteristics thought to occur only in rat mast cell protease type II. These results are in accord with the view that a protease cascade plays a key role in cytotoxic T-cell activation.

YTOTOXIC T LYMPHOCYTES (CTL'S), also referred to as T killer cells, are effector cells in cell-mediated immune reactions. They specifically recognize foreign antigens on the surface of target cells, bind to them, and cause the target cells to lyse. Although the various steps in this process have been analyzed in considerable detail (1, 2), most studies have not provided insight into the mechanism by which the killer cell effects the lysis of a target cell. A means of identifying relevant molecules, based on cloning CTL-specific genes, has been described (3). The transcripts corresponding to two of these genes (B10 and C11) were detected exclusively in activated CTL's (4). Moreover, the kinetics of messenger RNA (mRNA) expression, as detected by these two cloned probes, closely paralleled but preceded cytotoxicity throughout cytotoxic responses in vitro (3).

Sequence analysis (5) of B10 and C11 (Fig. 1A) revealed that they were related to each other and that the hypothetical proteins they encode contain a short region characteristic of serine proteases, Asp-Ser-Gly-Gly (a sequence homologous to that surrounding Ser¹⁹⁵ of chymotrypsin). With B10 and C11 as probes, another CTL complementary DNA (cDNA) library was screened, in which inserts greater than 1000 base pairs were cloned in λ gt10. Forty thousand recombinants were screened and 39 plaques corresponding to C11 were isolated, but no evidence for a B10 recombinant could be found.

A cDNA insert of 1400 base pairs, which hybridized with C11, was selected for sequence analysis (5). The predicted protein sequence encoded, of molecular weight 25,319, is shown in Fig. 1B. The putative start codon is preceded by a potential ribosome binding site CCUUCCG (6), and a polyadenylation signal sequence AAUAAA (7) occurs just upstream from the poly(A) tract. Of the first 12 amino acids predicted, ten are hydrophobic, and the amino acid in position 2 (Lys) is basic, suggesting that this sequence may act as a signal to direct secretion or intracellular organelle location (8).

A search of the National Biomedical Research Foundation (NBRF) protein sequence data bank revealed that the protein encoded by C11 resembles a number of serine proteases (Table 1). When the se-

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quences were optimally aligned according to the Dayhoff algorithm (9), the homologies generally varied between 30 and 40 percent. The greatest homology was found with rat mast cell protease type II (RMCPII), which had amino acids identical to 109 of 215 amino acids encoded by C11, giving a match per length of 51 percent. The amino acid residues known to form the catalytic triad of the active site in serine proteases (His⁵⁷, Asp¹⁰², and Ser¹⁹⁵) (10) were all found in the protein encoded by C11. The sequences around these residues, which are highly conserved among serine proteases, are also conserved in the C11 gene product. Indeed, largely because of conservation around this region, the protein encoded by Cl1 appears to be somewhat homologous (about 30 percent of 209 residues) even to the prokaryotic proteases trypsin and type B from Streptomyces griseus.

The cytotoxic T cell-specific proteins (CCP's) encoded by C11 and B10 will be referred to as CCPI and CCPII, respectively. In Fig. 2 the optimal protein alignment with CCPI is presented for RMCPII, bovine chymotrypsin, bovine trypsin, and CCPII. RMCPII is an intracellular serine protease found in the granules of atypical mast cells (11). The high level of homology of CCPI with RMCPII is particularly intriguing as RMCPII has a number of structural features that make it exceptional in the serine protease superfamily (12). Protein CCPI contains cysteines in precisely the same positions as RMCPII which, by analogy with RMCPII, form three disulfide bonds. These occur in the same positions in chymotrypsin, trypsin, and elastase. Both CCPI and RMCPII lack a disulfide bond that is present in all other known serine proteases, including several from prokaryotes (13), and that links Cys¹⁹¹ with Cys²²⁰ in chymotrypsin. In both CCPI and RMCPII the first of these two halfcystines is replaced by a phenylalanine, while the second half-cystine has been deleted along with other residues. Linkage of Cys¹⁹¹ to Cys²²⁰ is thought to be important in stabilizing the conformation of the substrate binding site (12). Its absence in CCPI and RMCPII may lead to significant changes in that site and, hence, in substrate specificity.

Two other primary structure changes previously seen only in RMCPII and thought to alter substrate binding are also present in the predicted CCPI protein. In RMCPII and CCPI the amino acid six residues before the active-site serine is alanine. In chymotrypsin-like proteases it is serine and in trypsin-like proteases, aspartic acid. The residue in this position lies at the bottom of the S_1 binding site (14), so the change to a less polar residue would indicate a preference for a hydrophobic amino acid at the P₁ position in the substrate (13). Furthermore, the sequence Ser-Trp-Gly²¹⁶ in chymotrypsin, which forms hydrogen bonds with the P1 and P_3 residues of the substrate (14), is replaced by Ser-Tyr-Gly in CCPI and RMCPII, again suggesting altered substrate specificity. Both of these changes are also seen with CCPII.

ences that is not present in CCPI is the substitution of isoleucine at position 99 in chymotrypsin for asparagine. In most mammalian serine proteases this residue is hydrophobic, and indeed in CCPI it appears to be phenylalanine. However, most of the RMCPII-specific changes are present in CCPI protein, suggesting that the substrate binding site of CCPI will resemble that of

One of the few RMCPII-specific differ-

B C

	A	
0 1	1 CARABACACTGCACGAAGTTAAGCTGACAGTACAGAAGGATCAGGTGTGTGAGTCCCCAGTTCCAAAGTTTTTACAACAGAGCTAATGAGATATGTGTC 485 CARACACGCTACAAGAGGTTGAGCTGACAGTACAGAAGGATCGGGAGTGTGAGTCCTACTTTAAAAATCGTTACAACAAAAAACCAATCAGATATGTGC * * * * * * * * *	3GG SGG
0	101 AGACTCAAAGATCAAGGGAGCTTCCTTTGAGGAGGATCCTGGAGGGCCGCTTGTGTGTAAAAGAGCAGCTGCAGGCATCGTCTCCTACGGGCAAACT	GAT
1	585 GGACCCAAAGACCAAACGTCCTTCCTTCGGGGGATTCNGAGGCCGCTGTGTGTGTGTAAAAAAGGCCGCAGGGATAGTTCCCTAGAATAAAA * * AspSerGlyGly	3AT
0	201 GGATCAGCTCCGCAAGTCTTCACAAGAGTTTTGAGTTTTGTATCGTGGATAAAGAAAACGATGAAACACAGGTAACTACAAGAAGCAAC TAGATCC	TG
1	685 GGTTCACCTCCACGTGCTTTCACCAAAGTCTCGAGTITCTTATCCTGGATAAAGAAAACAATGAAAAGCAGCTAACTAC AGAAGCAACATGGATCC	IGC *
10	299 ACTGA CAGCCATCTTCCC ATAGCTGAGTCCAGGATTGCTCTAGGACAGATGGCAGGCA	393 878
	B	
	GGCCTTCCCGGGAAGATGAAGATCCTCCTCGCTACTGCTGACCTTGTCTCTGGCCTCCAGGACAAAGGCAAGGGAGGATCATCGGGGGAACATGAAGTCAAGC MetLysIleLeuLeuLeuLeuLeuThrLeuSerLeuAlaSerArgThrLysAlaGlyGluIleIleGlyGlyHisGluValLysP	
	Sau 2 Sau 2 150 200	
	CCCACTCTCGACCCTACATCGCCTTACTTTCGATCAAGGATCASCGCCTGAGGCGATATGTGGGGGCTTCCTTATTCCAGAGGGGCTTTGTGCTGACTGC roHisSerArgProTyrMetAlaLeuLeuSerIleLysAspGlnGlnProGluAlaIleCysGlyGlyPheLeuIleArgGluAspPheValLeuThrAl	
	20 4U 250	
	TGCTCACTGTGAAGGAAGTATAATAAATGTCACTTGGGGGCCCCACAACATCAAAGAACAGGAGAAGACCCAGCAAGTCATCCCTATGGTAAAATGCATT aAlaHisCysGluGlySerIleIleAsnValThrLeuGlyAlaHisAsnIleLysGluGlnGluLysThrGlnGlnValIleProMetValLysCysIle	
	▲	
	CCCCACCCAGACTATAATCCTAAGACATTCTCCAATGACATCATGCTGCTAAAGCTGAAGAGTAAGGCCAAGAGGACTAGAGCTGTGAGGCCCCCAACC ProHisProAspTyrAspTroLysThrPheSorAsnAspIleMetleuLeuLysLeuLysSerLysAlaLysArgThrArgAlaValArgProLeuAsnL a0	
	TGCCCAGGCGCAATGTCAATGTGAAGCCAAGGAGATGTGTGTG	
	120 190 Sauta . 550 600	
	GGTTGACCTGACAGTACAGAAGGATCGGGAGTGTGACTCCTACTTTAAAAATCGTTACAACAAAAACCAATCAGATATUTGUGGGGGACCCAAAGACCAAA uValGluLeuThrValGlnLysAspArgGluCysGluSerTyrPheLysAsnArgTyrAsnLysThrAsnGlnIleCysAlaGlyAspProLysThrLys	
	160	
	b30 PSt1 CGTGCTTCCTTTCGGGGGGATTCTGGAGGCCCGCTTGTGTAAAAAGGGGCGGCAGGGATAGTTTCCTATGGATATAAGGATGGTTCACCTCCACGTG ArgAlaSerPheArgGlyAspSerGlyGlyProLeuValCysLysLysValAlaAlaGJyIleValSerTyrGlyTyrLysAspGlySerProProArgA	
	180 ▲ 200 750 BamHT . 800	
	CTTTCACCAAAGTC1CGAGTTTCTTATCCTGGATAAAGAAAACAATGAAAGCAGCTAACTACGGAGCAACATGGATCCTGCTCTGATTACCCATCGTC laPheThrLysValSerSerPheLeuserTrpIleLysLysThrMetLysSerSer***	
	220 900	
	SBUSA 830 CCTAGAGCTGAGTCCAGGATTGCTCTAGGACAGGTGGCAGGATCTGAATAAAGGACTGCAAAGACTGGCTTCATGTCCATTCACAAGGACCAGCTCTGTC	
	950 - 1000	
	1050 TTTGCATTGGAGCTGGGCATGCTCTGCTTCCCCTCAGTGCCCCGAGAATGTTATCTAATGCTAGTAGTCATTAATAGCTCCCTACAGAACTTTCATACAG	
	1150 EcoRI	
	11000000001101010101010101000000000000	
	1250 TGTATGTTTTATAAAGCTAATTTCCTTATCAAATGACATCTTTTAATTTTTACATTAATGGCTTATTTTCAAGGTACAACCTGATTTTTTATGGACAAA	
	Sau3a 1350 Αλτορηζοταλλητολληταλακοταλητή απηγτατος καταλαλαλαλαλαλαλ	

Fig. 1. (A) Nucleotide sequence comparison of clones B10 and C11. Insert DNA was purified from the CTL-specific clones designated B10 and C11 (3), recloned in M13 or pUC vectors and sequenced by the dideoxy method (5). The two sequences were maximally aligned; * designates nucleotide mismatches. The sequence of B10 is numbered from the first nucleotide of the insert, whereas that of C11 has been renumbered to correspond with the full-length sequence shown in (B). The region encoding the characteristic serine protease sequence Asp-Ser-Gly-Gly is boxed, and the polyadenylation signal sequence of B10 is underlined. (B) Nucleotide and predicted protein sequence of C11 insert. A size selected cDNA library (>1000 base pairs) was screened with a C11 insert isolated from the original library (3). A plaque that contained a cross-hybridizing sequence, whose apparent length was 1400 base pairs was selected for further analysis. Restriction fragments were subcloned in MI3 or pUC13 and sequenced as above. A potential initiator methionine codon is present at nucleotide 16 followed by an open reading frame to nucleotide 756. A putative ribosome binding site (-----) and a polyadenylation signal (----) are underlined. By analogy with RMCPII (see text and Fig. 2), the numbering of the C11 protein starts at the isoleucine 21 residues downstream from the proposed translational initiation site. The amino acids which form the catalytic triad of the serine proteases (His⁵⁷, Asp¹⁰², and Ser¹⁹⁵ in chymotrypsin) are marked (\blacktriangle).

Table 1. A selection of proteins that are homologous to the predicted C11 protein, CCPI. The protein sequence predicted from the longest open reading frame encoded by CCPI was compared with the National Biomedical Research Foundation protein sequence data bank. The numbering for CCPI (see text) is given in Fig. 1B. The data bank numbering system has been used for homologous proteins. All of the proteins that were significantly homologous (>30%) with CCPI over a large portion of the molecule (>150 residues) were serine proteases.

	E.C. number	Species source	Residues compared		Percent
Protein			ССРІ	Bank protein	homology
75 nerve growth factor	3.4.21	Murine	29-224	26-229	40
Chymotrypsin A	3.4.21.1	Bovine	1-200	16-216	35
Chymotrypsin B	3.4.21.1	Bovine	1-200	16-216	36
Complement C1r	3.4.21.41	Human	52-224	56-238	35
Elastase	3.4.21.11	Porcine	3-220	3-233	33
Factor X	3.4.21.6	Bovine	1-225	192-421	33
RMCPII	3.4.21	Rat	1-214	1-213	51
Kallikrein	3.4.21.8	Rat	26-225	51-262	36
Plasminogen	3.4.21.7	Human	3-224	563-787	37
Plasminogen activator	3.4.21.31	Human	72-224	389-560	35
Trypsin	3.4.21.4	S. griseus	29-220	22-214	33
Trypsin	3.4.21.4	Rat	29-226	31-228	39

CCPI RMCPII CA COW TR COW	Ile Ile Gly Gl Ile Ile Gly Gl Ile Val Asn Gl Ile Val Gly Gl	y His Glu Val y Val Glu Ser y Glu <u>Glu</u> Ala y Tyr Thr Cys	Lys Pro His Ser Ile Pro His Ser Val Pro Gly Ser Gly Ala Asn Thr	Arg Pro Tyr Met Arg Pro Tyr Met Trp Pro Trp Val Pro Tyr	Ala Leu Leu Scr Ile Lys Asp Ala His Leu Asp Ile Val Thr Gln Val Ser Leu Gln Asp Gln Val Ser Leu Asn Ser	22 22 37 26				
CCPI RMCPII CA COW TR COW	Gln Gln Pro Gl Glu Lys Gly Le Lys Thr Gly Pho Gly Tyr	u Arg Val Ile u Arg Val Ile e His Phe His Phe	Cys Gly Gly Phe Cys Gly Gly Phe Cys Gly Gly Ser Cys Gly Gly Ser	Leu Ile Arg Leu Ile Ser Arg Leu Ile Asn Leu Ile Asn Leu Ile Asn	Glu Asp Phe Val Leu Thr Ala Gln Phe Val Leu Thr Ala Glu Asn Trp Val Val Thr Ala Ser Gln Trp Val Val Ser Ala	42 43 55 44				
CCPI RMCPII CA COW TR COW	Ala His Cys Ala His Cys Ala His Cys Ala His Cys Ala His Cys Ty	Glu Gly Ser Lys Gly Arg Gly Val Thr r Lys Ser Gly	Ile 1le Asn Val Glu Ile Thr Val Thr Ser Asp Val Ile Gln Val	Thr Leu Gly Ile Leu Gly Val Val Ala Gly Arg Leu Gly	Ala His Asn Ile Lys Glu Gln Ala His Asp Val Arg Lys Arg Glu Phe Asp Gln Gly Ser Ser Gln Asp[Asn Ile] Asn Val Val	62 63 76 64				
CCPI RMCPII CA COW TR COW	Glu Lys Thr Glu Glu Ser Thr Gl Ser Glu Lys Il Glu Gly Asn Gl	n Gln Val Ile n Gln Lys Ile e Gln Lys Leu n Gln Phe Ile	Pro Met Val Lys Lys Val Glu Lys Lys Ile Ala Lys Ser Ala Ser Lys	Cys Ile Pro His Gln Ile Ile His Val Phe Lys Asn Ser Ile Val His	Pro Asp Tyr Asn Pro Lys Thr Glu Ser Tyr Asn Ser Val Pro Ser Lys Tyr Asn Ser Leu Thr Pro Ser Tyr Asn Ser Asn Thr	84 85 98 86				
CCPI RMCPII CA COW TR COW	Phe Ser Asn As Asn Leu His As Ile Asn Asn As Leu Asn Asn As	p Ile Met Leu p Ile Met Leu p Ile Thr Leu p Ile Met Leu	Leu l.ys Leu L.ys Leu l.ys Leu Glu Leu Lys Leu Ser Ile l.ys Leu Lys	Ser Lys Ala Lys Lys Lys Val Glu Thr Ala Ala Ser Ser Ala Ala Ser	Arg Thr Arg Ala Val Arg Leu Thr Pro Ala Val Asn Phe Ser Gln Thr Val Ser Leu Asn Ser Arg Val Ala	105 106 119 107				
CCPI RMCPII CA COW TR COW	Pro Leu Asn Le Val Val Pro Le Ala Val Cys Le Ser Ile Ser Le	u Pro Arg Arg u Pro Ser Pro u Pro Ser Ala u Pro Thr	Asn Val Asn Val Ser Asp Phe 11e Ser Asp Asp Phe Ser Cys Ala Ser	Lys Pro Gly Asp His Pro Gly Ala Ala Ala Gly Thr Ala Gly Thr	Val Cys Tyr Val Ala Gly Trp Met Cys Trp Ala Ala Gly Trp Thr Cys Val Thr Thr Gly Trp Gln Cys Leu Ile Ser Gly Trp	127 128 141 127				
CCPI CCPII RMCPII CA COW TR COW	Gly Ar Gly Ly Gly Leu Thr Ar Gly As	g Met Ala Pro s Thr Gly Val g Tyr Thr Asn n Thr Lys Ser	Met Gly Lys Arg Asp Pro Ala Asn Thr Ser[Gly]Thr Ser]	Tyr Ser Asn Thr Ser Tyr Pro Asp Tyr Pro Asp	Thr Leu Gln Glu Val Glu Leu Thr Leu His Glu Val [Lys] Leu Thr Leu Arg Glu Val Glu Leu Arg Leu Gln Gln Ala Ser Leu Val Leu Lys Cys Leu Lys Ala	145 146 160 146				
CCPI CCPII RMCPII CA COW TR COW	Thr Val Gln Ly Thr Val Gln Ly Arg Ile Met As Pro Leu Leu Se Pro Ile Leu Se	s Asp Arg Glu s Asp Gln Val p Glu Lys Ala r Asn Thr Asn r Asn Ser Ser	Cys Glu Ser Tyr Cys Glu Ser Gln Cys Val Asp Tyr Cys Lys Lys Tyr Cys Lys Ser Ala	Phe Lys Asn Phe Gln Ser Arg Tyr Tyr Glu Tyr Gly Thr Tyr Pro Gly	Arg Tyr Asn Lys Thr Asn Gln Phe Tyr Asn Arg Ala Asn Glu Tyr Lys Phe Gln Lys 11e Lys Asp Ala Met Gln Ile Thr Ser Asn Met	166 165 179 166				
CCPI CCPII RMCPII CA COW TR COW	Ile Cys Ala Gl Ile Cys Val Gl Val Cys Val Gl Ile Cys Ala Gl Phe Cys Ala Gl	y Asp Pro Lys y Asp Ser Lys y Ser Pro Thr y Ala Ser Gly y Tyr Leu Glu	Thr Lys Arg Ala Ile Lys Gly Ala Thr Leu Arg Ala Val Ser Ser Gly Gly Lys Asp	Ser Phe Arg Gly Ser Phe Glu Glu Ala Phe Met Gly Cys Met Gly Ser Cys Gln Gly	Asp Ser Gly Gly Pro Leu Val Asp Ser Gly Gly Pro Leu Val Asp Ser Gly Gly Pro Leu Leu Asp Ser Gly Gly Pro Leu Val Asp Ser Gly Gly Pro Val Val	188 187 200 188				
CCPI CCPII RMCPII CA COW TR COW	Cys Lys Lys Va Cys Lys Arg Al Cys Ala Gly Va Cys Lys Lys As Cys Ser Gly Ly	l Ala Ala a Ala Ala l Ala His n Gly Ala Trp s Leu Gln	Gly Gly Thr Leu Val Gly Gly	Ile Val Ser Tyr Ile Val Ser Tyr Ile Val Ser Tyr Ile Val Ser Trp Ile Val Ser Trp	Gly Tyr Lys Asp Gly Gly Gln Thr Asp Gly Gly Gln Thr Asp Ala Gly His Pro Asp Ala Gly Ser Ser Thr Cys Gly Ser Cly Cys Ala	204 203 222 205				
CCPI CCPII RMCPII CA COW TR COW	SerProPrSerAlaPrLysProPrSerThrPrLysAsnLysPrLysPr	o Arg Ala Phe o Gln Val Phe o Ala Ile Phe o Gly Val Tyr o Gly Val Tyr	Thr Lys Val Ser Thr Arg Val Leu Thr Arg Val Ser Ala Arg Val Thr Thr Lys Val Cys	Ser Phe Leu Ser Ser Phe Val Ser Thr Tyr Val Pro Ala Leu Val Asn Asn Tyr Val Ser	Trp Ile Lys Lys Thr Met Lys Trp Ile Lys Lys Thr Met Lys Thr Ile Asn Ala Val Ile Trp Val Gln Gln Thr Leu Ala Trp Ile Lys Gln Thr Ile Ala	225 223 243 227				
CCPI Ser Ser 227 CCPII His Ser RMCPII Asn 224 Fig. 2. Alignment of the predicted CCPI protein sequence with other sering CA COW Ala ASN 245 protessor. The protein sequence predicted from CLL is aligned according to										

^{CA} COW Ala Asn 245 TR COW Ser Asn 229 proteases. The protein sequence predicted from C11 is aligned, according to the method of Dayhoff (9), with rat mast cell protease (RMCPII), bovine chymotrypsin (CA COW), bovine trypsin (TR COW), and the partial protein sequence predicted from insert B10 (CCPII). Amino acids that are identical to those of CCPI are boxed. Residue numbers are given at the end of each line; CCPI and RMCPII start at the Ile residue, whereas the numbering of CA and TR is based on the inactive zymogens. No numbering is presented for CCPII because the full sequence has yet to be determined.

860

RMCPII and could be significantly different from those of other mammalian serine proteases (15).

In addition to the interesting aspects of the structure of CCPI, the fact that both CCPI and CCPII appear to be serine proteases has significant implications for the mechanism by which CTL's are activated to become capable of lysing target cells (1, 2). Protease inhibitors have been shown to block T-cell effector function (16), and a trypsin-like esterase activity was shown to be induced during CTL activation (17). We have demonstrated that two genes that encode protease-like proteins are expressed in CTL's. Previous evidence that these genes are activated specifically in CTL's and that their expression correlates with cytolytic activity (3) suggests that they play a key role in the development of a cytotoxic response.

The two genes we have so far characterized appear to encode serine esterases, and another CTL-specific gene, AR10, cloned by Gershenfeld and Weissman (18) encodes a trypsin-like enzyme. These findings suggest that a protease cascade mechanism may be involved in T-cell cytotoxicity. Such a mechanism has been shown in a number of biological systems, including blood coagulation and complement-mediated lysis, and is advantageous in control, specificity, and amplification of enzymatic reactions (19).

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The Locale Map of Honey Bees: Do Insects Have Cognitive Maps?

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Whereas higher vertebrates are able to construct a mental "map" of their home area and so use their knowledge of the spatial relations between landmarks to navigate along novel routes, invertebrates have been thought able to use landmarks in their navigation only as a familiar, route-specific series. Experiments with honey bees show that these insects have and use landmark maps thus invalidating this presumed invertebrate-vertebrate dichotomy.

LTHOUGH HONEY BEES USE CELEStial cues in their navigation to unfamiliar food sources (1), they frequently begin to rely on prominent landmarks, at least on the outward journey, as the route becomes familiar (2, 3). Wehner (4) has proposed that the landmark memory of invertebrates is stored as a series of routespecific photographs; hence, bees and other insects could use landmarks only in serial order and along familiar routes. This model stands in sharp contrast to the way higher vertebrates are thought to use landmarks as part of a map; in this system, the relative location of familiar landmarks is understood-presumably stored in the brain as a map-so that novel routes based on new combinations of landmarks may be used, freeing the animal from dependence on route-specific combinations.

To put this presumed vertebrate-invertebrate dichotomy into concrete terms, consider the area around an animal's home (Fig. 1). If the hive is its home and site A is a food source, an insect would be presumed to know site A in terms of a set of landmarks regularly encountered there; if displaced to site B, the insect would, at best, recognize site B as part of some other route leading from home to another food source, and perhaps be able to follow that route back home. A higher vertebrate familiar with the same area, on the other hand, would be able to use the landmarks visible at site B to determine the direction of site A, and set off directly toward it even though it had never traveled from site B to site A before; the

vertebrate, it is supposed, has integrated its landmark knowledge into a map.

Two observations suggest that the map alternative might be available to bees. First, older foragers captured while leaving the hive, transported in darkness to a location hundreds of meters away and out of sight of the hive, fed a highly concentrated sugar



Fig. 1. Bees from a hive (bottom center) were trained to either site A, site B (both 160 m from the hive), or (off the top of the map) site C (350 m from the hive); on subsequent days these foragers and others were captured and transported to a different site and released-site B for those trained to A, site A for those trained to B, and site D (350 m from the hive) or site E (4425 m from the hive) for those trained to site C. As a control, some trained foragers were released at site H as well. Arrow N marks north.

solution, and released can fly directly home (5, 6); that young foragers cannot suggests that some familiarity with the locale may be important. This result is not conclusive since the foragers might have recognized the release site as part of their route-specific memory.

The second suggestion comes from experiments in which forager dances were manipulated to indicate a location either in the middle of a lake or on the far shore (7,8). Recruitment was effective only to the latter, more plausible site, suggesting that the dance coordinates enabled recruits to make some sort of judgment about the suitability of the advertised site before leaving the hive; recruits make economic judgments about the distance versus the quality of the food being advertised before leaving (9). But the lake results could be explained on the basis of environmental factors (8). The purpose of the experiments reported here was to determine whether bees are limited to route-specific navigation or, instead, have a true locale map available to them.

Individually marked honey bees (Apis mellifera ligustica) were first trained to site A 160 m west of the hive (Fig. 1) along a path through the woods at Stony Ford, the Princeton University field station in Princeton, New Jersey. On the basis of the training procedure and the observed departure bearings from both the hive and the food source, foragers flew either directly to and from the hive to reach the feeding station or along a two-legged route that included the last portion of the training route (the path). All bees visiting the station were either marked or captured.

On subsequent days, foragers regularly visiting site A were captured at the hive entrance in a 600-ml beaker placed in their takeoff path. Each trapped forager was then carried in the dark across an open field to site B, 160 m south-southwest of the hive, and released. Two foragers were sometimes captured and transported together, but bees

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