730 element (base pairs 101 through 383 fused to 619 through 730; Fig. 2A) (*11*). Binding site 6 for TLL(181-517) was obtained with a different subfragment of the *Kr* 730 element and is not shown in Fig. 1A. TLL(181-517) binding sites were confirmed by use of a TLL(181-517) expression plasmid that produced the full-length TLL protein (data not shown).

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15. The consensus sequences for KNI and TLL(181-517) were established from footprints of the regulatory regions of three KNI and TLL target genes. Fourteen binding sites were used to generate the KNI binding consensus (AA/TCTAA/GATC), and fifteen binding sites were used to generate the TLL(181-517) consensus (AAATTAA). M. Hoch, C. Hartmann, H. Jäckle, unpublished results.
16. *Drosophila* Schneider line S2/M3 cells were grown [P. P. Di Nocera and I. B. David, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7095 (1983)], plated, and transfected as described (*28*). For transfection, a total of 20 μg of DNA containing various amounts of effector plasmid DNA, 1 μg of reporter plasmid DNA, 3 μg of pPac*lacZ* reference plasmid DNA [see (*17*) and Fig 3], and Bluescript carrier DNA (Stratagene) were used. Transfected cells were grown for 60 hours, washed once with phosphate-buffered saline (PBS), and lysed by repeated freeze-thaw cycles. Cell debris was removed by centrifugation. The supernatant was assayed for β-galactosidase activity to account for variations of transfection efficiency. CAT assays were performed as described [C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982)]. The production and nuclear localization of KNI and BCD in transfected cells were controlled by antibody stainings (data not shown).
17. For construction of the *Kr* 16–*lacZ* reporter gene, a double-stranded oligonucleotide of the sequence 5′-ACTGAACTAAATCCGG-3′, derived from the *Kr* 730 element (Figs. 1 and 2B), was synthesized. After self-ligation, a six-copy fragment (five sites in tandem orientation, the last one in opposite orientation) was cloned into the KrZ P-element vector (*11*). The reporter plasmid p*Kr*16AdhCAT was constructed by insertion of seven copies of the 16-bp element (five in tandem orientation and two in opposite orientation; see above) into the unique Xba I site of the pPAdh86 vector [described in (*28*)]. The pPac*kni* effector plasmid was constructed by blunt ligation of a 2.5-kb Nru I fragment of the *kni* coding region into the Bam HI site of the pPac vector (*28*). The pPacTN*bcd* effector plasmid is a pPac derivative (*27*). The pPac*lacZ* reference plasmid contains the *lacZ* coding region followed by the SV40 polyadenylation signal of the pCaSpeR AUGβ-gal vector [C. S. Thummel, A. M. Boulet, H. D. Lipshitz, *Gene* **74**, 445 (1988)].
18. The fusion gene construct *Kr* 16–*lacZ* was integrated into the *Drosophila* genome by P-element–mediated germline transformation [G. M. Rubin and A. C. Spradling, *Science* **218**, 348 (1982)]. DNA constructs were injected into *w* sn^{w} homozygous mutant embryos. Transformant lines were established, and their embryonic progeny was assayed for *lacZ* expression [Y. Hiromi and W. J. Gehring, *Cell* **50**, 963 (1987)] with antibody staining (*19*). For each experiment, at least three independent transformant lines were analyzed.
19. Antibody stainings of whole mount embryos were carried out as described [P. Macdonald and G. Struhl, *Nature* **324**, 537 (1986)] with the Vectastain ABC Elite-horseradish peroxidase system (Vector Laboratories). For staining conditions and staining control experiments see (*11*). For the staining pattern of *even-skipped* see M. Frasch and M. Levine, *Genes Dev.* 1, 981 (1987).
20. Females containing extra copies of the *bcd* gene were derived from the fly strain *bcd*$^{+5/+8}$ described (*21*). Maternally mutant embryos were scored as described (*11*); the mutant strain *bcd*^{E1} has been described (*12*). The hsp70*kni* flies contain the *kni* coding region fused to an inducible hsp70 promoter; hsp70*tll* flies contain the *tll* coding region fused to an inducible hsp70 promoter. Embryos carrying the *kni* hsp70 P-element or the corresponding *tll* hsp70 P-element were heat-shocked as described (*4, 24*). Heat-shocked embryos were identified by staining with an anti-eve antibody (*19*).
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Molecular Cloning of the Interleukin-1β Converting Enzyme

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Interleukin-1β (IL-1β) mediates a wide range of immune and inflammatory responses. The active cytokine is generated by proteolytic cleavage of an inactive precursor. A complementary DNA encoding a protease that carries out this cleavage has been cloned. Recombinant expression in COS-7 cells enabled the cells to process precursor IL-1β to the mature form. Sequence analysis indicated that the enzyme itself may undergo proteolytic processing. The gene encoding the protease was mapped to chromosomal band 11q23, a site frequently involved in rearrangement in human cancers.

The cytokine interleukin-1 (IL-1) has been implicated in inflammation, septic shock, and other physiological situations, including wound healing and the growth of certain leukemias (*1*). There are two distantly related forms of IL-1 (*2*), IL-1α and IL-1β; the latter is the predominant species released by monocytes. Most efforts to develop an IL-1 antagonist have focused on inhibition of binding to the IL-1 receptor, which mediates biological responses to both forms of the cytokine (*3*). In some circumstances, it may be desirable to discriminate between the two forms of IL-1; an alternative approach specific to IL-1β involves the inhibition of the protease required for its biosynthesis. This protease (the IL-1β converting enzyme or convertase) cleaves the inactive 31-kD precursor of IL-1β between Asp^{116} and Ala^{117}, releasing the 153 COOH-terminal amino acids that constitute the mature cytokine (*4–6*). The critical function of this protease is indicated by the recent finding that cowpox virus encodes a highly specific inhibitor of the enzyme; this inhibitor is necessary for the virus to suppress the host inflammatory response (*7*). To pursue the study of this protease, we have cloned a cDNA that encodes a proteolytically active form of this converting enzyme.

The IL-1β converting enzyme was purified from the human acute monocytic leukemia cell line THP-1, and 20 amino acids from the NH_2-terminus were determined (Fig. 1) (*8*). The convertase cDNA was isolated in three stages. First, single-stranded cDNA prepared from THP-1 cells was amplified by polymerase chain reaction (PCR) with degenerate oligonucleotide primers based on the NH_2-terminal sequence (*9*). This action resulted in a cDNA

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  1  MADKVLKEKRKLFIRSMGEGTINGLLDELL
 31  QTRVLNKEEMEKVKRENATVMDKTRALIDS
 61  VIPKGAQACQICITYICEEDSYLAGTLGLS
 91  ADQTSGNYLNMQDSQGVLSSFPAPQAVQDN
121  PAMPTSSGSEGNVKLCSLEEAQRIWKQKSA
151  EIYPIMDKSSRTRLALIICNEEFDSIPRRT
181  GAEVDITGMTMLLQNLGYSVDVKKNLTASD
211  MTTELEAFAHRPEHKTSDSTFLVFMSHGIR
241  EGICGKKHSEQVPDILQLNAIFNMLNTKNC
271  PSLKDKPKVIIIQACRGDSPGVVWFKDSVG
301  VSGNLSLPTTEEFEDDAIKKAHIEKDFIAF
331  CSSTPDNVSWRHPTMGSVFIGRLIEHMQEY
361  ACSCDVEEIFRKVRFSFEQPDGRAQMPTTE
391  RVTLTRCFYLFPGH
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Fig. 1. Deduced amino acid sequence (*23*) of the human IL-1β converting enzyme encoded by the cDNA inserts p1bp, p48, and p214 (*9–11*). The DNA sequence has been submitted to GenBank under accession number M87507. Amino acids determined by sequence analysis of the purified protein are underlined (*8*).

clone that encoded the 16 NH_2-terminal amino acids of the purified protease. Next, we used 3′-anchored PCR (*10*) to isolate a cDNA of ~1000 bp (p1bp) (Fig. 1). This clone encoded 285 amino acids, including all of the residues determined by protein sequencing (*8*). Finally, a probe derived from the anchored PCR clone was used to screen a cDNA library prepared from human peripheral blood neutrophils (*11*). This action resulted in the isolation of two clones, p48 and p214, with inserts of 1366 and 1359 bp, respectively.

As deduced from these clones, the IL-1β convertase cDNA is 1373 bp in length, including a stretch of adenine (A) residues corresponding to the polyadenylated [poly(A)$^+$] tail of the mRNA. These A residues are preceded by two polyadenylation signals (AATAAA) at 1316 and 1335 bp. The sequence has an open reading frame (ORF) encoding 404 amino acids (Fig. 1), starting with an initiator methionine codon at nucleotide (nt) 18 and ending with a termination codon at nt 1230 (*12*). Initiation of translation could also begin with an in-frame methionine codon at nt 66. Both of these methionine codons have consensus Kozak translation initiation sequences (*13*). The NH_2-terminus of the purified protein corresponds to Asn^{120}.

To confirm that the protein encoded by these cDNAs can convert precursor IL-1β to the mature form, we inserted cDNA from p48 into a mammalian expression vector and cotransfected it into COS-7 cells with a second mammalian expression plasmid that contained a cDNA encoding precursor IL-1β. After 2 days, cells were labeled with ^{35}S and both precursor and mature IL-1β were immunoprecipitated from cell lysates and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography (*14*). The COS-7 cells processed precursor IL-1β to the mature form when they were cotransfected with a plasmid encoding IL-1β convertase, but not if they were cotransfected with control plasmids (Fig. 2A). Cells transfected with a plasmid encoding a form of the convertase that lacked the first 119 amino acids of the ORF were also able to process precursor IL-1β to the mature form. To ensure that the processed form of IL-1β began with Ala^{117}, we cotransfected COS-7 cells as described above, except that the cells were labeled with [^{3}H]leucine, and processed IL-1β was radiosequenced (*15*). Peak counts of tritium were found in cycles 6, 10, and 18, which corresponded to the positions of leucine residues in authentic IL-1β with an NH_2-terminus of Ala^{117} (Fig. 2B).

Analysis of the amino acid sequence encoded by the convertase cDNA showed no similarity to any known protein (GenBank, release 70.0). Because its inhibitor sensitivity suggests that the convertase is a cysteine protease (*4*), we focused particular attention on areas around cysteine and histidine residues that form the active site of cysteine proteases. We found no similarity to any known cysteine proteases or to any proteases of the other mechanistic classes. Hydrophobicity analysis showed no lengthy hydrophobic domains, consistent with the cytoplasmic location of the protease. Because the NH_2-terminus of the enzyme purified from THP-1 cells corresponds to Asn^{120} (Fig. 1), and the molecular size of the purified convertase is 22 kD as determined by SDS-PAGE (*8*), it appears that the protease undergoes both NH_2-terminal and COOH-terminal processing. It is possible that processing is autocatalytic because aspartic acid, the primary determinant of the substrate specificity of the convertase (*16*), is at positions 119 and 297. Cleavage at these sites could yield the form of the protein purified previously (*8*).

Although we have demonstrated that the 119 NH_2-terminal amino acids are not required for activity in transfected COS-7 cells (Fig. 2A), the function of the COOH-terminal region is less clear. Plasmid constructions that delete portions of the COOH-terminal region as well as the 119 NH_2-terminal amino acids yielded proteins without detectable processing activity (*17*). The importance of the COOH-terminal domain is supported by analysis of human and murine convertase cDNAs (*18*), which have a 62% amino acid identity; the COOH-terminal domains have an 80% identity. The COOH-terminal domain could be required for proper folding of the active site, possibly as a second subunit.

We performed Northern (RNA) blot analysis with a variety of RNA preparations to investigate the expression of the IL-1β convertase gene. Transcripts of ~2500, ~1900, and ~500 nt were detected in THP-1 cells; the 1900-nt transcript was the most abundant (Fig. 3A). The cDNAs that we have isolated probably correspond to this transcript. The convertase mRNA was detected in a variety of cells including peripheral blood monocytes, peripheral blood lymphocytes, peripheral blood neutrophils, resting and activated peripheral blood T lymphocytes, placenta, and the B

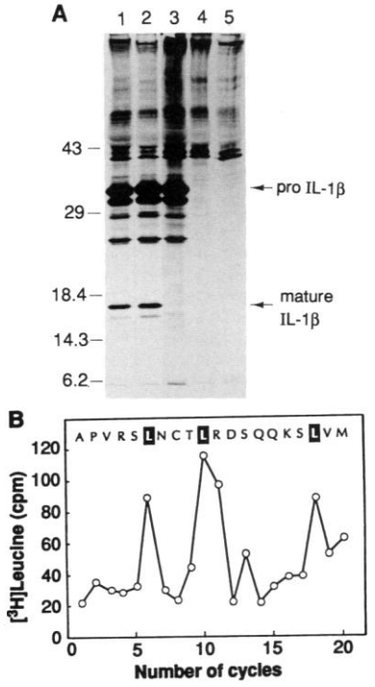

Fig. 2. Recombinant expression of IL-1β convertase. (**A**) Autoradiogram of proteins immunoprecipitated from transfected COS-7 cells with antisera to IL-1β. COS-7 cells were transfected with the following plasmids (5 μg): lane 1, precursor IL-1β/CAV and 1bp/CAV; lane 2, precursor IL-1β/CAV and FL1bp/CAV; lane 3, precursor IL-1β/CAV only; lane 4, CAV (empty vector); lane 5, 1bp/CAV only. Two days after transfection, cells were labeled, and proteins were immunoprecipitated with an IL-1β–specific antiserum and subjected to SDS-PAGE before autoradiography (*14*). The locations of precursor IL-1β (pro IL-1β) and mature IL-1β are indicated. Molecular size standards in kilodaltons are indicated to the left. (**B**) Radiosequencing of the processed IL-1β protein from transfected COS-7 cells. Cells were transfected as described for lane 2 in (A) except that proteins were labeled with [^{3}H]leucine. The processed IL-1β protein was purified and radiosequenced as described (*15*). The data shown are representative of two experiments. The amino acid sequence at the top is the NH_2-terminus of mature human IL-1β (*2, 23*).

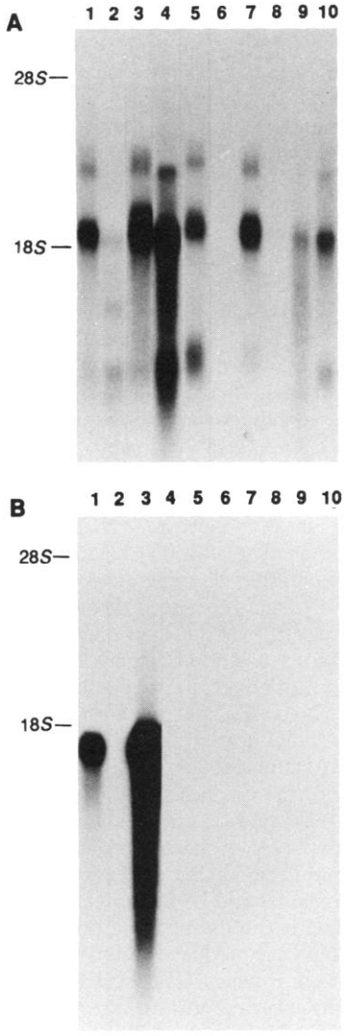

Fig. 3. Northern blots hybridized with a probe for human IL-1β convertase (**A**) or human IL-1β (**B**). Filters contain human poly(A)$^+$ RNA (2.5 μg) except for neutrophils and HepG2, for which total RNA was used (2.5 μg). Lane 1, peripheral blood monocytes; lane 2, peripheral blood lymphocytes; lane 3, THP-1; lane 4, peripheral blood neutrophils; lane 5, placenta; lane 6, HepG2; lane 7, CB23; lane 8, Raji; lane 9, resting peripheral blood T cells; lane 10, activated peripheral blood T cells. The cell lines and the methods for RNA isolation and Northern blot analysis were as described (*25*). Antisense probes were used for human IL-1β convertase and human IL-1β (Promega). The positions of 18*S* and 28*S* ribosomal RNA are indicated to the left.

lymphoblastoid line CB23. Another B lymphoblastoid line, Raji, as well as HepG2 cells had no detectable convertase mRNA. Reprobing the blot for IL-1β mRNA (Fig. 3B) showed that only peripheral blood monocytes and THP-1 cells had detectable amounts of this mRNA. The tissue distribution of the convertase suggests that the enzyme may have other substrates in addition to precursor IL-1β.

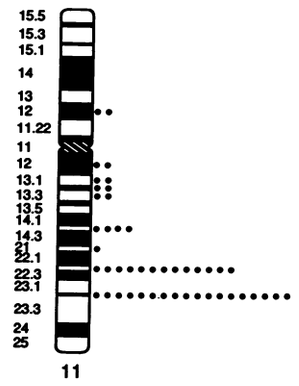

Fig. 4. Distribution of labeled sites on chromosome 11 from normal metaphase peripheral blood lymphocytes that were hybridized with a ^{3}H-labeled IL-1β convertase probe. A total of 226 chromosomally localized grains were scored over 104 metaphases (*20*). Each dot represents two autoradiographic grains. Forty-two percent (95 out of 226) of grains localized in 11q with no grain clusters in any other chromosome region. Sixty-six percent (63 out of 95) of the 11q grains were over region 11q13 to 11q23.2, with the highest concentration of grains at 11q23.2.

The IL-1β convertase gene was located on chromosome 11q13-11q23 with Southern (DNA) blot analysis of rodent-human hybrids (*19*) and on chromosome 11q23 by in situ hybridization to normal human metaphase preparations (Fig. 4) (*20*). Chromosome band 11q23 is frequently involved in rearrangement in human cancers, including a number of leukemias and lymphomas (*21*), and recent studies have implicated IL-1β as an autocrine growth factor in certain acute and chronic myelocytic leukemias (*22*). The finding that the convertase gene maps to band 11q23 raises the possibility that altered production of the protease contributes to some of these disease states.

The molecular cloning of a protease that generates active IL-1β provides new insight into IL-1 biology and offers a new target for the development of therapeutic agents. The amino acid sequence confirms that the convertase is unrelated to any known protease, which increases the likelihood that specific inhibitors can be developed.

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9. C. Lee and C. T. Caskey, in *PCR Protocols*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, New York, 1990), pp. 46–53. Two pools of oligodeoxynucleotide primers were used: 5′-GTCGAATTCAA(T,C)CC(A,C,G,T)-GC(A,C,G,T)ATGCC(A,C,G,T)AC-3′ (sense), which included all possible codons specifying amino acids NPAMPT (*23*) and an Eco RI restriction site and 5′-GTCTCTAGAAG(T,C)TT(A,C,G,T)AC(A,G)-TT(A,C,G,T)CC(T,C)TC-3′ (antisense), which included sequences complementary to all possible codons specifying amino acids EGNVKL and an Xba I restriction site (*8*). mRNA was isolated (*24*) from THP-1 cells cultured as described (*4*). Single-stranded cDNA (Amersham cDNA synthesis kit) was amplified with 4 mM solutions of each primer in 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM tris HCl (pH 8.3), 0.01% gelatin, 200 mM deoxynucleotide triphosphate (dNTP), and 1 U of *Thermus aquaticus* polymerase (Perkin-Elmer Cetus). The PCR was carried out with a denaturing temperature of 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min for 30 cycles. The PCR products were analyzed by PAGE (8%), and DNA was visualized by ethidium bromide staining. A 63-bp fragment was isolated, subcloned, and sequenced. Dideoxy sequencing was performed as described [D. P. Cerretti *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3223 (1986)]. Sequence analysis was aided by University of Wisconsin Genetics Computer Group sequence analysis programs [J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984)].
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11. A human neutrophil cDNA library (D. P. Cerretti, C. J. Kozlosky, T. Vanden Bos, N. Nelson, D. Gearing, M. P. Beckmann, in preparation) was screened as described (*24*).
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14. The cDNA inserts from p48 and p1bp were ligated into the mammalian expression vector pDC303 [B. Mosley *et al.*, *Cell* **59**, 335 (1989)], which resulted in plasmids FL1bp/CAV and 1bp/CAV, respectively. Plasmids (10 μg) were transfected into subconfluent monolayers of COS-7 cells and metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine as described [A. E. Namen *et al.*, *Nature* **333**, 571 (1988)]. Cells were lysed and IL-1β was immunoprecipitated with a rabbit antiserum. Proteins were analyzed by SDS-PAGE [U. K. Laemmli, *Nature* **227**, 680 (1970)]. The gels were treated with Enhance (DuPont Biotechnology Systems) and dried before autoradiography.
15. Radiolabeling and immunoprecipitation were carried out as above, except that [^{3}H]leucine (Amersham, 142 Ci/mmol, 0.1 Ci/ml) was used, and unlabeled human IL-1β (5 μg) was added before electrophoresis. After electrophoresis, the proteins were transferred to a polyvinyl difluoride membrane (ProBlott, Applied Biosystems) [P. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987)] and stained with Coomassie blue. The IL-1β band was excised from the membrane and loaded on an Applied Biosystems model 477A protein sequencer. The radioactivity released during each cycle of the sequencer was determined by scintillation spectroscopy.
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23. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Balancing Selection at Allozyme Loci in Oysters: Implications from Nuclear RFLPs

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Population genetic analyses that depend on the assumption of neutrality for allozyme markers are used widely. Restriction fragment length polymorphisms in nuclear DNA of the American oyster evidence a pronounced population subdivision concordant with mitochondrial DNA. This finding contrasts with a geographic uniformity in allozyme frequencies previously thought to reflect high gene flow mediated by the pelagic gametes and larvae. The discordance likely is due to selection on protein electrophoretic characters that balances allozyme frequencies in the face of severe constraints to gene flow. These results raise a cautionary note for studies that rely on assumptions of neutrality for allozyme markers.

Starch gel electrophoresis of soluble proteins has been the workhorse technique of population genetics for nearly 30 years. Although several studies have indicated that natural selection acts on particular allozyme loci (*1*–*6*), the working hypothesis of most population genetic applications has been that the majority of enzyme (as well as DNA) polymorphisms evolve as predicted by neutrality theory and can be interpreted accordingly for purposes of estimating population structure, gene flow, and genetic relatedness. Previous studies of the American oyster (*Crassostrea virginica*) in the southeastern United States revealed a remarkable contradiction between data from biparentally inherited allozyme loci, indicating little or no population subdivision (*7*), and maternally transmitted mitochondrial DNA (mtDNA), demonstrating a sharp genetic discontinuity between oyster populations from Atlantic Coast in contrast to Gulf of Mexico locales (*8*). Similar mtDNA surveys of a variety of coastal-restricted taxa, including horseshoe crabs, toadfish, black sea bass, diamondback terrapins, and seaside sparrows (*9*), demonstrate phylogenetic discontinuities between Atlantic and Gulf populations of these species, suggesting that similar historical processes are involved and that these biogeographic factors probably operated on American oysters as well.

There are several possible explanations for the apparent inconsistency between nuclear and cytoplasmic genetic structures in oysters. The discrepancy could be due to biological or demographic factors: (i) a higher rate of interpopulation gene flow mediated by sperm rather than by eggs; (ii) directional selection favoring different mtDNA haplotypes in the two regions; or (iii) a smaller effective population size for mtDNA that resulted in a faster rate of lineage sorting from the ancestral gene pool than was the case for most nuclear alleles (*10*). Alternatively, the apparent contradiction between the mitochondrial and nuclear genomes could be due to the following factors: (i) hidden variation within electromorph classes (cryptic allozymes), such that undetected allozyme differences truly distinguish Atlantic and Gulf populations; (ii) a slower rate of evolutionary change in allozyme frequencies; or (iii) balancing selection at multiple allozyme loci.

To distinguish between these two classes of competing hypotheses, we report here an analysis of restriction fragment length polymorphism (RFLP) in single-copy nuclear (scn) DNA. We constructed primers suitable for amplification of DNA by the polymerase chain reaction (PCR) for each of four anonymous nuclear loci (*11*), following a procedure described elsewhere (*12*). Nuclear DNA, isolated from each of 277 oysters collected at nine locations between Massachusetts and Louisiana (*13*), was amplified with the use of these primers (*14*). The amplified products were digested with restriction enzymes (*15*), and four restriction site polymorphisms interpretable as unlinked Mendelian variants at single genes were identified (*16*).

Restriction site polymorphisms from all four scn loci reveal significant shifts in allele frequency between the Gulf of Mexico and Atlantic collections of American oysters, with the differences between these two geographic regions generally much greater than those within either area (Table 1). The pattern contrasts strikingly with the geographic uniformity evidenced by the allozyme polymorphisms (Fig. 1). The pronounced genetic break in scnDNA appears along the eastern coast of Florida, as was true for the mtDNA phylogeographic break in oysters and other maritime species (*8*, *9*). The sample from Stuart, Florida, a geographically intermediate locale, generally exhibited transitional allele frequencies. A clustering of genetic distances based on the scnDNA allele frequencies further documents the dramatic population genetic separation between the Atlantic and the Gulf of Mexico oysters, the agreement with the mtDNA break, and the apparent contrast with the allozyme information (Fig. 2).

The similar pattern of geographic population subdivision registered in mtDNA and

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