The *Drosophila saxophone* Gene: A Serine-Threonine Kinase Receptor of the TGF-β Superfamily

Ting Xie, Alyce L. Finelli, Richard W. Padgett*

The *Drosophila decapentaplegic* (*dpp*) gene encodes a transforming growth factor–β (TGF-β)–like protein that plays a key role in several aspects of development. Transduction of the DPP signal was investigated by cloning of serine-threonine kinase transmembrane receptors from *Drosophila* because this type of receptor is specific for the TGF-β–like ligands. Here evidence is provided demonstrating that the *Drosophila saxophone* (*sax*) gene, a previously identified female sterile locus, encodes a TGF-β–like type I receptor. Embryos from *sax* mothers and *dpp* embryos exhibit similar mutant phenotypes during early gastrulation, and these two loci exhibit genetic interactions, which suggest that they are utilized in the same pathway. These data suggest that *sax* encodes a receptor for *dpp*.

The TGF-β superfamily comprises an ancient group of genes with members in both vertebrates and invertebrates. TGF-β–like peptides are secreted regulatory molecules that control the growth or differentiation of cells (*1*). The mechanisms by which TGF-β–like family members effect these changes are poorly understood. The first member to be identified from invertebrates, *dpp* (*2*), has been extensively studied. It is involved in a variety of developmental decisions and plays a key role in dorsal-ventral polarity of the *Drosophila* embryo (*3*, *4*). Later in development, *dpp* is involved in gut formation and in patterning the adult animal (*3*, *4*). How *dpp* and other TGF-β–like genes mediate communication between cells and effect developmental change remains largely unknown.

Components of the TGF-β–like signaling system are functionally conserved in evolutionarily distinct phyla. This is best demonstrated by the observation that sequences encoding the ligand domain of the human bone morphogenetic protein 4 (BMP-4) can substitute for the corresponding domain of the *Drosophila dpp* gene in flies (*5*) and rescue null *dpp* embryos to viability. Like the BMPs, *dpp* can induce the formation of cartilage and bone in rats (*6*). This functional conservation suggests that components identified in one organism are likely to be similar in other organisms.

The identification of receptors for members of the TGF-β–like family provides an entry point to unravel the signal transduction pathway. In vertebrates, cross-linking experiments reveal the presence of three membrane-associated proteins, betaglycan and type I and type II receptors, that tightly bind TGF-β–like molecules (*7*). Betaglycan has been postulated to present the ligand to the receptor, rather than to directly mediate downstream biological responses (*8*). The type I and type II receptors are transmembrane serine-threonine kinases (*9*, *10*). The type II receptor can be chemically cross-linked to the growth factor ligands and is responsible, at least in part, for transducing the signal (*9*–*11*). Type I and II heterodimers mediate most, if not all, of the biological activity of this pathway (*11*, *12*). The exact role of each type of serine-threonine kinase receptor in transducing the signal is not well understood. Few of the characterized intracellular signaling components for the well-studied tyrosine kinase receptors are known to act in the TGF-β–like pathway (*13*).

To understand how TGF-β–like molecules communicate information between cells, it is necessary to isolate and characterize the components of the signal transduction pathway. Because *dpp* plays a critical role in several developmental processes in *Drosophila*, we focused on cloning candidate receptors for *dpp*, none of which has been identified. We designed degenerate primers in order to isolate candidate receptors by polymerase chain reaction (PCR). The primers correspond to the conserved cytoplasmic domains of the serine-threonine kinase receptors of activin and TGF-β (*9*). Positive clones were isolated and analyzed as described (*14*).

One clone, Rec43E, contains an open reading frame of 570 amino acids (Fig. 1). Rec43E has a putative NH_2-terminal signal peptide with a consensus site for proteolytic cleavage after amino acid 21 (*15*), which leaves a mature polypeptide of 549 residues with a mass of 62 kD. The putative protein has a hypothetical transmembrane domain (amino acids 177 to 199). The predicted ectodomain is cysteine-rich, a characteristic common to ectodomains of receptors of the TGF-β superfamily ligands. The pattern of cysteines in the putative ectodomain of Rec43E has little similarity with other TGF-β–like receptors. Their spacing may be important for determining the three-dimensional structure of the ectodomain and, consequently, ligand specificity.

Several pieces of evidence indicate that Rec43E is a type I receptor (Fig. 2). Sequence comparisons of amino acid sequences of the known type I and type II receptors show that Rec43E is more similar to type I receptors than to type II receptors. There are several highly conserved regions in the kinase domains of all type I receptors, which are absent in all the type II receptors. Type I receptors contain the following conserved sequences: the GSGS box, the EIF box, the RIKKT box, and a shortened COOH-terminal tail (Fig. 2). The Rec43E sequence contains these conserved type I domains. Furthermore, dendrogram analysis indicates that Rec43E is more related in overall amino acid sequence to known type I receptors than to type II receptors (Fig. 3). In the protein kinase domain, the type I receptors average 78%

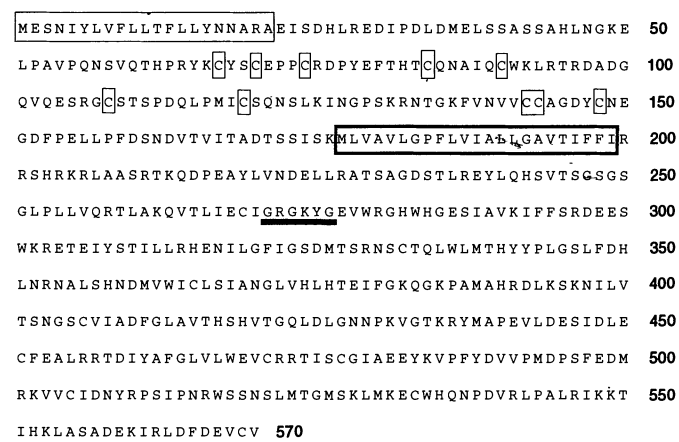

Fig. 1. The deduced amino acid sequence of the *sax* cDNA (*25*). Amino acid sequences are numbered to the right of each sequence. The putative NH_2-terminal hydrophobic signal sequence and the 10 cysteine residues in the ectodomain are denoted by open boxes. A predicted transmembrane domain is indicated by a bold open box, and the consensus ATP-binding site is underlined. The Rec43E cDNA was isolated from a 3- to 12-hour embryonic library (*26*) and sequenced as described (*2*, *23*). The nucleotide sequence of *sax* has been deposited with GenBank (accession number U05209).

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identity between each other and share only 49% identity with the type II receptors.

Rec43E is located at position 43E on the *Drosophila* polytene chromosome map (*16*). Examination of the *Drosophila* database (*17*) revealed that the *sax* gene is also located in this region. The *sax* gene was isolated in a screen for recessive female sterile genes on the second chromosome by Schüpbach and Wieschaus (*18*). Of the loci identified in screens for female steriles, *sax* is one of the few genes that affects the dorsal-ventral axis whose role has not been established (*18, 19*). The *sax* gene is included in the dorsal-ventral group because of its early gastrulation defects. In particular, embryos from *sax* mothers have a deep dorsal cephalic furrow, and their germband does not extend to its full length dorsally (*18*). These phenotypic defects are similar to the defects previously described for *dpp* (*3, 4*) (Fig. 4). Similar mutant phenotypes would be predicted for *dpp* and its receptor. Unlike *dpp*, the embryonic expression of *sax* mRNA is not spatially localized in the embryo, although the two expression patterns overlap temporally and spatially and are expressed in the same cells (*16*). The specific defects exhibited by *sax* embryos can be explained if their mutant phenotypes are determined by the absence of reception of a spatially localized ligand. From the similarity of embryonic defects seen in *sax* and *dpp* embryos, we hypothesized that *sax* encodes Rec43E and is a *dpp* receptor.

To determine if *sax* indeed corresponds to Rec43E, we cloned the Rec43E sequences from the two existing mutant *sax* strains and their isogenic parental strains. Because the *sax* mutations arose in a recessive female sterile screen that required homozygous animals to eclose, we expected that these *sax* alleles would be hypomorphic or antimorphic. If so, the sequence of mutant *sax* alleles would be expected to contain missense amino acid changes, rather than nonsense changes. As predicted, amino acid missense changes were found in both *sax* mutant chromosomes, which are different from the sequences of the isogenic parental chromosomes (*20*). Both *sax* alleles encode missense mutations that map near the receptor adenosine triphosphate (ATP) binding site, as determined by comparison to the three-dimensional structure of cy-

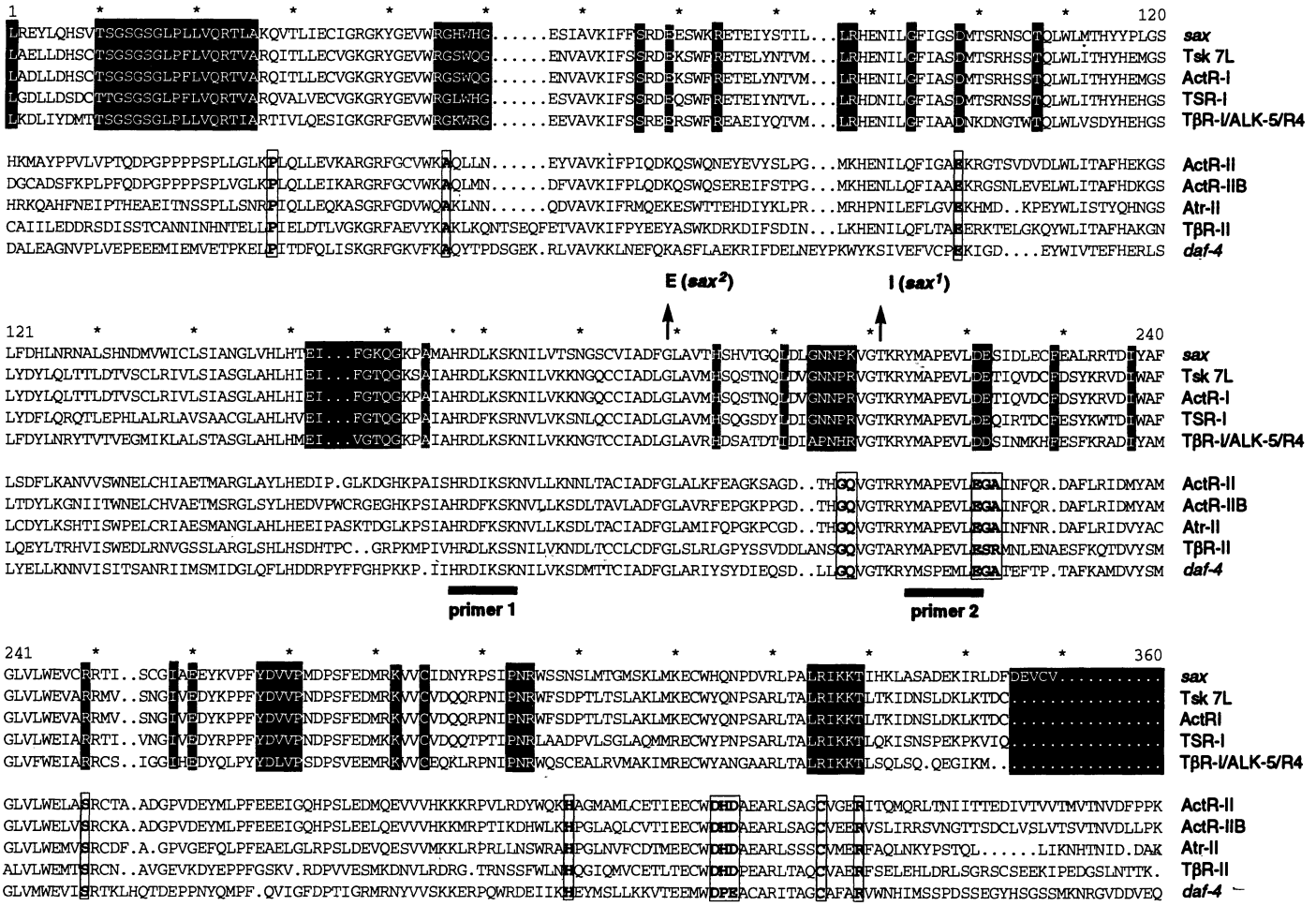

Fig. 2. Comparison of amino acid sequences of the kinase domain of type I and type II receptors of the TGF-β superfamily (*25*). The comparison begins approximately 20 amino acids after the transmembrane domain and was aligned by software in the Genetics Computer Group package (*27*). The top line of each block of amino acid sequence is numbered. Asterisks indicate intervals of 10 amino acids, and dots represent gaps used to generate optimal alignments. Sequences included in this figure have been shown by biochemical cross-linking studies to be either type I or type II receptors. The sources for the amino acid sequences are mouse Tsk 7L (*10*), human ActR-I (*10*), human TSR-I (*10*), human TβR-I/ALK-5 (*10*), rat R4 (*10*), mouse ActR-II (*9*), mouse ActR-IIB (*29*), *Drosophila* Atr-II (*28*), human TβR-II (*9*), and a *Caenorhabditis elegans* type II receptor, *daf-4* (*9*). The R4 sequence is identical to TβR-I and ALK-5 sequences in the kinase domain and may represent the rat homolog of these genes. The missense mutations of the two *sax* alleles, *sax*^{1} *and sax*^{2}, are indicated at positions 189 and 211, respectively. The type I receptors are listed on top and are separated from the type II receptors by a space. Boxes are placed around amino acids that are unique to either type I receptors (filled boxes) or type II receptors (open boxes), but are not common to both classes. The underlined sequences indicate regions in the kinase domain used to generate degenerate primers for amplification of receptor sequences (*14*).

clic adenosine monophosphate (cAMP)–dependent protein kinase (*21*) (Fig. 2). From this analysis, we conclude that *sax* encodes the Rec43E protein and is likely a serine-threonine kinase receptor of the TGF-β superfamily.

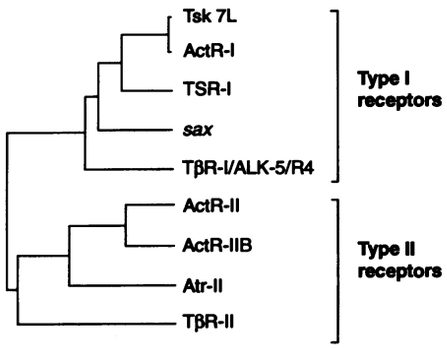

Fig. 3. Relation between the type I and type II serine-threonine kinase receptors in the kinase domain. The Genetics Computer Group software package (*27*) was used to produce a dendrogram that shows the relation of the receptors on the basis of sequence similarity. Similarity is indicated by the horizontal distance of any two sequences from a branch point. All the other genes in this diagram have been shown to be type I or type II receptors by biochemical cross-linking analysis. The dendrogram shows that *sax* is more similar to the type I receptors in overall homology than to the type II receptors.

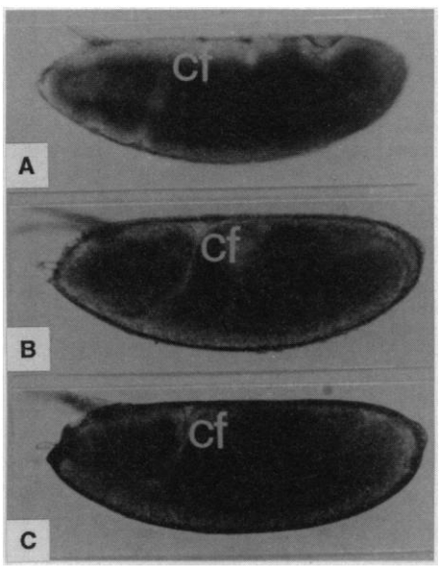

Fig. 4. Gastrulation phenotypes of living embryos. (**A**) A wild-type embryo. (**B**) A dpp^{H61} homozygous, null embryo. (**C**) Offspring from a *sax* mother. The dpp^{H61} allele contains a deletion of part of the coding sequences and is a *dpp* null allele (*30*). Because sax^1/sax^1 or sax^2/sax^2 offspring occur infrequently in our stocks, we examined offspring from sax^1/sax^2 mothers. The cephalic furrow (cf) of mutant *dpp* embryos and embryos from *sax* mothers is deep on the dorsal side, indicating similar defects during gastrulation. Eggs were collected from each strain, placed in halocarbon oil, and observed under a miroscope in transmitted light with bright-field optics (*31*).

The similar phenotypes of *dpp* and embryos from homozygous *sax* mothers suggest that *sax* could be a *dpp* receptor. Genetic interactions between *dpp* and *sax* were examined to provide additional evidence for this model. A genetic interaction would suggest that the two genes participate in the same developmental pathway. A moderate allele of *dpp*, dpp^{hr27}, was tested for genetic interactions with *sax*. This *dpp* allele was used previously to characterize genetic interactions between *dpp* and *tolloid*, a gene that acts to modify *dpp*'s activity before receptor binding (*22*, *23*). Heterozygous *sax* females were crossed to heterozygous dpp^{hr27} males and the progeny scored. Adult offspring carrying either sax^1 or sax^2 in combination with dpp^{hr27} (sax/dpp^{hr27}) do not eclose, thus indicating a strong genetic interaction with both alleles (Table 1). This interaction is not specific for dpp^{hr27}; similar genetic interactions are observed with dpp^{hr93} (*16*).

Table 1. Genetic interaction between *sax* and *dpp*. Male flies of the genotype dpp^{hr27}/CyO (*17*) were crossed to virgin females of genotype (i) *Df(2R)pk78s* (deficiency for *sax*), (ii) sax^1/CyO, (iii) sax^2/CyO, and (iv) *cn bw* (parental stock). Adult progeny were scored for the presence or absence of *Cy*, a dominant adult marker present on the *CyO* balancer chromosome. Because *CyO/CyO* animals die before eclosion, one-third of the adults in this cross are expected to carry *sax* and *dpp* mutant alleles if *sax* and *dpp* do not genetically interact. The ratio of the number observed to the number expected is expressed as a percentage. The sax^1 and sax^2 alleles exhibit antimorphic characteristics as compared to the *sax* deficiency.

♀♀	dpp^{hr27} ♂♂		
	Number scored	Number expected	Number observed (%)
cn bw	264	88	97 (110%)
Df(2R) pk78s	342	114	32 (28%)
sax^1/CyO	407	136	0 (0%)
sax^2/CyO	136	45	0 (0%)

Furthermore, we tested the ability of *dpp* duplications to rescue embryos derived from homozygous *sax* mothers, which are normally sterile. Because the *sax* mutations appear to be partial loss of function alleles, increasing the dosage of *dpp* might result in viable animals. Two transgenic lines, carrying different *dpp* transposons, were used to increase the dosage of *dpp* (*17*). We observed that all of the offspring that survived to adulthood contained one copy of the *dpp* transposon, in addition to the two genomic copies of *dpp* (Table 2). The ability of an extra copy of *dpp* coding sequences to rescue these embryos further supports the model that they are involved in a common regulatory pathway.

Table 2. Rescue of embryos from homozygous *sax* mothers by a duplication of *dpp*. Two different *dpp* transposons were used in examining the ability to rescue embryos from homozygous *sax* mothers to viability. P6.5 (*17*) is a *dpp* transposon on the second chromosome and is balanced by the *CyO* balancer chromosome. Homozygous *sax* females were crossed to P6.5/*CyO* males. A control cross with the parental strain, *cn bw*, indicates that P6.5 produces *Cy* and Cy^+ offspring at the same frequency (*16*). The only offspring that survive are Cy^+ animals, which carry two genomic copies of *dpp* and one copy of the *dpp* transposon, indicating that the *dpp* transposon is required for viability. P20 strains, which carry a 20-kb *dpp* transposon on the X chromosome (*17*), normally produce both male and female progeny (*16*). Homozygous *sax* females were crossed to P20 males, and the sex ratio of offspring was scored. Only female offspring were recovered, whereas no male offspring were produced, indicating that the *dpp* transposon is required for viability.

♂♂	*sax/sax* ♀♀		
	Number scored	Number expected	Number observed (%)
P6.5/*CyO*	128	64	128 (100% Cy^+)
P20/—	107	54	107 (100% females)

The inclusion of *sax* in the *dpp* pathway and the identification of *sax* as a putative TGF-β–like type I receptor further our understanding of how the dorsal-ventral pattern is established in *Drosophila*. The existence of a type I receptor for the *dpp*-BMP family indicates that this family of ligands transduces signals through a type I and type II receptor complex, like the activin and TGF-β families. Because the type I and type II receptors function together to transmit signals to the nucleus of the cell, it will be critical to identify the type II receptor (or receptors) involved in *dpp* signaling. Through genetic and biochemical means, the *sax* receptor will provide an avenue for identifying the type II receptor (or receptors) and the downstream components of the *dpp* signaling pathway. There are many vertebrate homologs of *dpp* that have the potential to specify cell fates (*1*). The isolation of vertebrate *sax* homologs will be an important step in understanding how these growth factors mediate signaling.

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14. To amplify DNA from the kinase domain of TGF-β–like receptors, we made primers corresponding to two conserved regions of known kinases (Fig. 2). The sequences of the primers are as follows: primer 1, CA(C/T) CG(A/C/G/T) GA(C/T) AT(A/C/T) AA(A/G) TC(A/C/G/T) AA(A/G) AA; and primer 2, TC(A/C/G/T) AG(A/C/G/T) A(C/T)(C/T) TC(A/C/G/T) GG(A/C/G/T) GCC AT(A/G) TA. Genomic DNA and DNA from a 4- to 8-hour embryonic complementary DNA (cDNA) library (*24*) were amplified by PCR and cloned into pBluescript (Stratagene), which had been treated with Eco RV, TAQ polymerase, and dTTP. Conditions for PCR were as follows: three cycles at 94°C for 1 min, 40°C for 1 min, 72°C for 3 min, followed by 27 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Random colonies were sequenced (*2, 23*), analyzed by computer, and placed into groups on the basis of sequence identity. After all the clones were compared and grouped, cDNA clones were isolated from the positive inserts and sequenced to verify that they belonged in the TGF-β–like receptor family.
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Protection from Fas-Mediated Apoptosis by a Soluble Form of the Fas Molecule

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Fas is an apoptosis-signaling receptor molecule on the surface of a number of cell types. Molecular cloning and nucleotide sequence analysis revealed a human Fas messenger RNA variant capable of encoding a soluble Fas molecule lacking the transmembrane domain because of the deletion of an exon encoding this region. The expression of soluble Fas was confirmed by flow cytometry and immunocytochemical analysis. Supernatants from cells transfected with the variant messenger RNA blocked apoptosis induced by the antibody to Fas. Levels of soluble Fas were elevated in patients with systemic lupus erythematosus, and mice injected with soluble Fas displayed autoimmune features.

The Fas/Apo-1 molecule (designated as CD95) is a cell-surface receptor belonging to the nerve growth factor receptor–tumor necrosis factor–α receptor family of apoptosis-signaling molecules (*1*). Defects in the mouse *Fas* gene lead to autoimmune features in mice (*2*), and the human Fas molecule has been implicated in the abnormally high levels of lymphocyte apoptosis seen in human immunodeficiency virus (HIV)–infected humans (*3*). To investigate the possible presence of abnormal Fas transcripts in human subjects with autoimmune disease, we isolated cellular RNA from the peripheral blood mononuclear cells (PBMC) of two systemic lupus erythematosus (SLE), two angioimmunoblastic lymphadenopathy (AILD) patients, and two normal controls. The Fas mRNA transcripts were then analyzed after amplification with the use of oligonucleotide primers derived from the 5′ untranslated region (UTR) starting at nucleotide (nt) 170 and 3′ UTR and ending at nt 1336 (Fig. 1A). The amplified product was expected to be 1167 base pairs (bp) in size and to encompass the entire translation region, as well as portions of the 5′ and 3′ UTRs of the Fas mRNA. However, in addition to the anticipated full-length Fas complementary DNA (cDNA) fragment, a distinct smaller 1104-bp DNA fragment was also observed in patients with SLE and AILD and in normal controls.

The structure of the smaller polymerase chain reaction (PCR) product was determined by cloning (Fig. 1B), DNA se-

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