A Candidate Gene for the amnionless Gastrulation Stage Mouse Mutation Encodes a TRAF-Related Protein

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We report the identification of a new recessive prenatal lethal insertional mutation, amnionless (amn). amn mutant embryos first appear abnormal during the Early Streak stage, between E6.5 and E7.0, when they initiate mesoderm production. Subsequently, the amn mutants become developmentally arrested between the Mid and Late Streak stages of gastrulation and they die and are resorbed between E9.5 and E10.5. While extraembryonic structures, including the chorion, yolk sac blood islands, and allantois appear to develop normally, the small embryonic ectoderm remains undifferentiated and generates no amnion. In addition, the embryonic mesoderm that is produced does not become organized into node, notochord, and somites and there is no morphological evidence of neural induction. Interspecific backcross and fluorescence in situ hybridization analyses map the transgene insertion, and thus the amn mutation, to the distal region of mouse chromosome 12, which has synteny with human chromosome 14q32. A gene encoding a 7.5-kb transcript has been identified at a junction between the integrated transgene and host chromosome 12 sequences that meets three criteria expected of a candidate amn gene. This gene maps to the site of transgene insertion; it is transcribed during gastrulation, and its expression is disrupted in amn mutant embryos. Nucleotide sequencing studies show that the 567 amino acid protein encoded by the 7.5-kb transcript is a member of the newly defined family of putative signal transducing proteins, TRAFs, that associate with the cytoplasmic domains of members of the tumor necrosis factor (TNF) receptor superfamily. Thus, we have named the gene encoding the 7.5-kb transcript TRAFamn. TRAFamn is identical to a recently reported protein (CD40bp, CAP-1, CRAF1, LAP1) that can bind the cytoplasmic domains of CD40 and the lymphotoxin β receptor (LTβR), both of which are known members of the TNF receptor superfamily. The implications of these findings regarding a possible role for the TNF receptor superfamily during gastrulation are discussed.

INTRODUCTION

Gastrulation is a fundamental developmental process that coordinates complex cell and tissue movements with cell growth and proliferation to reorganize and differentiate the embryonic ectoderm into the three definitive germ layers of the fetus: ectoderm, mesoderm, and endoderm (Gilbert, 1991). Gastrulation also establishes the basic body plan of the embryo and generates the germ layer interactions required for subsequent tissue differentiation during organogenesis. In the mouse, gastrulation takes place during the early postimplantation period of development, between the Pre Streak stage at approximately Embryonic Day (E) 6.0 and the Headfold stages at E 7.5–8.0 (Lawson and Pedersen, 1987; Downs and Davies, 1993).

A comparison of the fate maps for the mouse Pre...
Early Streak embryo (Lawson et al., 1991) with those for Xenopus, chick, and teleost gastrula shows that the topographical organization of the prospective tissue lineages is remarkably similar among the four organisms and suggests that the molecular mechanisms mediating gastrulation are conserved among vertebrate species (Beddington and Smith, 1993; Beddington, 1994; Lawson et al., 1991; Quinlan et al., 1995). In Xenopus, tissue manipulations and RNA/protein injection experiments have identified several different molecules that can influence mesoderm formation, axis development, and neural tissue differentiation, including the transcription factor goosecoid, the secreted protein noggin, Wnt-related growth factors, and members of the transforming growth factor (TGF)-β and fibroblast growth factor (FGF) families and their receptors (Slack, 1994; Kelly and Melton, 1995). Consistent with the hypothesis that similar molecules direct gastrulation in different vertebrate species, recent studies have shown that mouse, chick, and zebrafish homologs of a number of these molecules are expressed in conserved patterns during gastrulation (Beddington and Smith, 1993; Slack, 1994).

The functions of a number of these molecules during mouse gastrulation have now been investigated by the generation of germ line null mutations. Interestingly, while the targeted inactivation of some of these mouse genes has led to predicted defects in gastrulation (fibroblast growth factor receptor 1, Fgfr-1; Yamaguchi et al., 1994, and Deng et al., 1994), the inactivation of others has not generated gastrulation stage mutations (Fgf-4: Feldman et al., 1995; Fgf-5: Herbert et al., 1994; goosecoid, Rivera-Perez et al., 1995, and Yamada et al., 1995; and activins, Matzuk et al., 1995a,b). The latter outcome may result from a requirement for a gene prior to gastrulation, as in the case of Fgf-4 (Feldman et al., 1995), from the presence of other genes with redundant functions, from the availability of protein synthesized by maternal tissues, or from a lack of function during mammalian gastrulation.

Another strategy to genetically define gastrulation in the mouse has been to characterize spontaneous, irradiation-induced, and transgene-generated insertional mutations that produce gastrulation stage defects (Herrmann and Kispert, 1994; Holdener-Kenny et al., 1992; and Conlon et al., 1994). An attractive feature of this approach is that it begins with a germ line mutation that disrupts gastrulation. Thus, the mutated gene must play a nonredundant role in mouse gastrulation and zygotic expression of the mutated gene is not essential for the pre- and implantation stages of development. Interestingly, this strategy has yielded genes not previously identified in the Xenopus system, including Brachyury (T), which plays a critical role in the ingress of nascent mesoderm (Herrmann and Kispert, 1994) and nodal, which is required for the formation and maintenance of the primitive streak and which may also be needed for the establishment and maintenance of the node (Conlon et al., 1994).

In the present study, we report the identification of a new transgene-induced insertional mutation, amnionless (amn), which disrupts gastrulation in the mouse. Embryos homozygous for the transgene insertion first appear abnormal during the Early Streak stage, between E6.5 and E7.0; subsequently they die and become resorbed between E9.5 and E10.5. Our histological analyses indicate that both the growth and differentiation of embryonic ectoderm cells are severely and specifically impaired. We also describe the identification of a candidate for the disrupted gene at the amn locus that is responsible for the gastrulation stage defects. This gene, TRAFamn, encodes a member of a new family of putative signal transducing proteins that associate with the cytoplasmic domains of receptors in the tumor necrosis factor (TNF) superfamily. This superfamily of receptors functions in coordinating the survival, proliferation, and differentiation of lymphocytes following stimulation by antigen and cytokines. Intriguingly, the amn mutant appears to be defective in supporting the survival, proliferation, and differentiation of embryonic ectoderm cells during streak formation and elongation. These findings raise the possibility that members of the TNF receptor superfamily are involved in coordinating the growth and differentiation of the gastrulating mouse embryo.

MATERIALS AND METHODS

Histological Analyses

Embryos were dissected from the uteri of pregnant females as described by Hogan et al. (1994). For histological analyses, embryos in their surrounding decidua were fixed overnight in Bouin’s solution and stored in 70% ethanol. Following ethanol dehydration and clearing in toluene or xylene, the decidua were embedded in paraffin (a 1:1 mixture of Paraplast and Histoplast; Fisher Scientific). Seven-micrometer sections were prepared and stained with hematoxylin and eosin.

Preparation and Screening of T81-3 Genomic Libraries

Junction 2 (x2n2) between the T81 transgene and flanking mouse sequences was cloned from a total genomic T81-3 library in λDASH II (Stratagene). Genomic DNA for the library was prepared according to Mark et al. (1992). For preparation of the T81-3 total genomic library, DNA isolated from the kidneys of a T81-3 heterozygote was partially digested with Ndel, fractionated on a sucrose step gradient, and the fraction containing fragments averaging 20 kb in length was ligated into the CIP-treated BamHI sites of the λDASHII arms. About 1.5 × 10⁶ plaque forming units (PFU) from nine independent packagings with either Gigapack GOLDII or Gigapack XLII (Stratagene) were screened with a human CD8α cDNA (Littman et al., 1985); only one of these packagings was amplified prior to screening 10⁶ PFU. In total, 30 positive clones were isolated. All plasmid subclones were constructed and purified using standard procedures (Maniatis et al., 1989).

Genotyping Embryos from amn/+ Intercrosses

Whole E7.5–E9.5 embryos were genotyped by the PCR assay diagrammed in Fig. 5, which uses three primers flanking the transgene insertion site at jxn2. Primer PF is from flanking host
sequences, primer PRT from the transgene, and primer PR from host sequences displaced by the inserted transgene. Wild-type embryos generate a 180-bp PCR product (PF and PR) and homozygous mutant embryos generate a 119-bp PCR product (PF and PRT), whereas heterozygous embryos produce both the 180- and 119-bp PCR products.

The embryos were dissected from the maternal decidua and Reichert's membrane in Hank's balanced salt solution supplemented with 10% fetal calf serum and then were placed individually in 10-50 ml of PCR-proteinase K buffer as described by Yamaguchi et al. (1994). After an overnight incubation at 55°C, the embryo lysates were boiled for 10 min to inactivate the proteinase K. Subsequently, 2-5 ml of each embryo lysate was added to 20-30 ml of 20% Chelex, incubated at 37°C for 1 hr, and boiled for 10 min. Each 50-ml PCR reaction contained 5-10 ml of the Chelex mixture, 0.8 U Taq DNA polymerase (Boehringer-Mannheim), and 73 ng each of PF and PR and 37 ng of PRT in 2.5 mM MgCl2, 200 mM dNTP. The reactions were run for 43 cycles of 94°C for 30 sec, 58.4°C for 30 sec, and 72°C for 45 sec; the PCR products were resolved by electrophoresis through 2.5% Seakem LE agarose gels. A full-length composite chromosome Preparation Northern Blot Analysis

Interspecific Mouse Backcross Analysis

Interspecific backcross progeny were generated by mating C57BL/6j x M. spreptus females and C57BL/6j males as described (Copeland and Jenkins, 1991). A total of 205 N2 mice were used to map the amn locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins et al., 1982). All blots were prepared with Hybond N+ nylon membrane (Amersham). Two mouse genomic probes were used in these studies. The first probe (E3a-3.2, Fig. 5), an approximately 3.2-kb XbaI fragment of mouse genomic DNA, was labeled with [α-32P]dCTP using a nick translation labeling kit (Boehringer-Mannheim); washing was done to a final stringency of 0.2× SSC, 0.1% SDS, 65°C. A fragment of 3.4 kb was detected in XbaI-digested C57BL/6j DNA and a fragment of 4.7 kb was detected in XbaI-digested M. spreptus DNA. A second probe (p1.1/Jxn2, Fig. 5), an approximately 1.1-kb XbaI fragment of mouse genomic DNA, produced fragments of 8.2 (C57BL/6j) and 5.2 kb (M. spreptus) following digestion with HindIII. The presence or absence of the 4.7-kb XbaI and 5.2-kb HindIII sperlocus-specific fragments, which cosegregated, was followed in backcross mice. The data here were generated from the E3a-3.2 probe.

A description of the probes and the restriction fragment length polymorphisms (RFLPs) for the loci linked to amn, including α-1-antitrypsin (Aat) and immunoglobulin in heavy chain complex (Ighc), has been reported previously (Dickinson et al., 1990; Singh et al., 1991). Recombination distances were calculated as described (Green, 1981) using the computer program Spretus M A D N E S S. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Fluorescence in Situ Hybridization (FISH) and Chromosome Preparation

A mouse genomic probe containing the human CD8α transgene T8I, a 10.4-kb gene fragment cloned into pSP64, was labeled with biotin-11-dUTP by nick translation (Gibco BRL, Gaithersburg, MD). The size of the products were determined to be between 200 and 400 bp. Metaphase chromosome preparations were derived from lymphocytes of a male amn/+ mouse. The hypotonic buffer was 0.075 M KCl and the fixative was methanol:acetic acid (3:1, v/v). Conventional G-banding was used for karyotyping. The hybridization was carried out as described by Edelhoff et al. (1994). Hybridization signals were detected using immunological reagents from Vector Laboratories (Burlingame, CA). After incubation with goat anti-biotin antibody, slides were rinsed in modified 2× SSC, 0.1% Tween 20, and 0.15% bovine serum albumin. A second incubation with fluorescein-labeled rabbit anti-goat IgG and a rinse in modified 2× SSC followed. The chromosomes were banded using Hoechst 33258-actinomycin D staining and counterstained with propidium iodide. The chromosomes and hybridization signals were visualized by fluorescence microscopy, using a dual band pass filter (Omega, Braitleboro, VT).

cDNA Cloning and Sequencing

A full-length composite T R A F α m n cDNA was cloned by screening three different mouse brain cDNA libraries. First, using a α-32P-labeled 1.1-kb XbaI fragment as probe, an oligo(dT)-primed C57BL/6j brain cDNA library (Stratagene) was screened using standard protocols (Maniatis et al., 1989). Second, a BALB/c brain 5'-stretch cDNA library (Clontech) was hybridized to the most 5' 0. 8 kb (nt 1897-2735) of the C57BL/6J cDNA fragment cloned in the first screen. Third, a BALB/c brain 5'-stretch plus cDNA library (Clontech) was screened using as probe the most 5' 0.6 kb (nt 871-1392) of the cDNA fragments isolated from the brain 5'-stretch library. cDNA fragments were cloned into pBluescript II (KS+) (Stratagene). The nucleotide sequence of the T R A F α m n cDNA was determined on both strands by dyeoxy sequencing using the U.S.B. Sequenase kit (version 2.0). The sequence data were analyzed using the program Macvector (Eastman Kodak Company). The nucleotide sequence of the T R A F α m n cDNA has been submitted to the GenBank database, Accession No. U 33840.

DNA Isolation

Adult mouse tissues and staged embryos (after removal of the visceral and parietal yolk sacs) were collected. Individual tissues and pools of embryos (E8.5-E17.5) were lysed in 4 M guanidine thiocyanate, 0.5 Na-laurylsarcosine, 25 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol, 0.1% Antifoam A (Sigma). The tissue lysates were layered over a 5.7 M CsCl cushion, and total RNA was precipitated by ultracentrifugation.

Mutant E7.5 embryos were collected and pooled from amn/+ intercross matings for total RNA preparation. Although the extraembryonic regions of both mutant and M idStreak normal embryos are about the same size at E7.5, the embryonic ectoderm of the mutant embryo is shortened compared with that of the normal embryo and could thus be identified. For cultured cells and mutant and C57BL/6j E7.5 embryos, total RNA was prepared using a single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction as described by Chomczynski and Sacchi (1987).

Northern Blot Analysis

Twenty micrograms of total RNA per sample was electrophoresed in a 0.8% agarose-formaldehyde gel and transferred to nylon membrane (Hybond NYTRAN, Schleicher & Schuell). T3 or T7 probes hybridized with 10× enzyme-labeled probes were prehybridized for 2 hr at 42°C, hybridized for 16 hr at 42°C, washed in 2× SSC, 0.1% SDS, and 0.15% bovine serum albumin, and finally washed once in 0.2× SSC at 55°C. The blots were exposed to Biomax MR film (Eastman Kodak Company) for 2-4 hr. The hybridization signals were detected using immunological reagents from Vector Laboratories (Burlingame, CA). After incubation with goat anti-biotin antibody, slides were rinsed in modified 2× SSC, 0.1% Tween 20, and 0.15% bovine serum albumin. A second incubation with fluorescein-labeled rabbit anti-goat IgG and a rinse in modified 2× SSC followed. The chromosomes were banded using Hoechst 33258-actinomycin D staining and counterstained with propidium iodide. The chromosomes and hybridization signals were visualized by fluorescence microscopy, using a dual band pass filter (Omega, Braitleboro, VT).
RNA polymerase in vitro transcribed sense or antisense\(^{32}\)P-labeled RNA probes were generated from a pBluescript (II/KS\(^{+}\)) subclone containing the 1.1-kb XbaI Jx2 fragment. Hybridization was performed overnight at 55°C in a buffer containing 50% formamide, 50 mM Na\(_2\)HPO\(_4\) (pH 8.0), 1% SDS, 5× SSC, 2.5× Denhardt’s, 250 \(\mu\)g/ml salmon sperm DNA, and 250 \(\mu\)g/ml yeast total RNA. Northern blots were washed at room temperature, one time for 15 min in 5× SSC, 0.05% SDS, then at room temperature one time for 15 min in 1× SSC, 0.1% SDS, and finally at 70°C one time for 15 min in 0.1× SSC, 0.1% SDS. Blots exhibiting high background were treated with RNase. Following a 30-min wash at room temperature in 2× SSC, the blot was incubated with 2 \(\mu\)g/ml of RNase A (Sigma) and 1 U/ml RNase T1 (Boehringer-Mannheim) at room temperature for 12 min and then washed for 15 min at 70°C in 0.1× SSC, 0.1% SDS. Hybridization signals were visualized by autoradiography using Kodak AR-5 X-ray film.

**In Situ Hybridization**

The 1.9-kb open reading frame of TRAFamn (nt 513–2505) was subcloned into pBluescript (II/KS\(^{+}\)). \(\text{[\text{p}]}\)UTP-labeled antisense and sense riboprobes were synthesized with the T3 and T7 RNA polymerases, respectively. In situ hybridization to 5-\(\mu\)m paraffin sections was carried out essentially as described (Manova et al., 1990) using 50% formamide.

**RNase Protection**

Twenty micrograms of total RNA from adult tissues, total RNA isolated from 42 dissected mutant E7.5 embryos, and total RNA dissected from 20 C57BL/6J E7.5 wild-type embryos were used for each assay. To make the 400-nt antisense TRAFamn probe, an EcoRV–XbaI fragment (nt 3093–3504) was cloned into pBluescript (II/KS\(^{+}\)). The plasmid was linearized with XhoI and transcribed with T7 polymerase, producing a probe which protects 155 bases of the TRAFamn transcript. To make the 600-nt antisense probe, a pBluescript (II/KS\(^{+}\)) subclone containing a 600-bp H\(/\)J600 cDNA fragment (Lee et al., 1992) was linearized with BamHI and transcribed with T3 polymerase, producing a probe that protects 155 bases of the H\(/\)J58 transcript. Both probes were of the same specific activity. Probes were hybridized with RNA samples overnight at 48°C in 1× RNase protection hybridization buffer (40 mM Pipes, pH 6.4, 0.4 M NaCl, 1 mM EDTA, pH 8.0) and then treated with 0.12 U/ml of RNase T1 (Boehringer-Mannheim) and 40 ng/ml RNase A (Sigma) for 1 hr at 30°C. Protected fragments were separated on 5% acrylamide–7 M urea gels and exposed to film for 2–4 days.

**Zoo Blot Analysis**

For the nonmammalian species, 10 \(\mu\)g of genomic DNA were digested with EcoRI, electrophoresed in a 0.8% agarose gel, and transferred to supported nitrocellulose BA–85 (Schleicher & Schuell). The filter was hybridized overnight with 10 ng/ml of probe, at 65°C, in a buffer containing 5× SSC, 2× Denhardt’s, 2.5 mM EDTA, 100 \(\mu\)g/ml sonicated denatured salmon sperm DNA. The blot was washed twice for 30 min in 2× SSC, 1× Blot Wash (25× Blot Wash contains 0.3 M Na\(_2\)HPO\(_4\), 0.23 M Na\(_2\)HPO\(_4\), 0.03 M Na\(_2\)HPO\(_4\), and 1.25% SDS) at 65°C, then one time for 10 min, in 1× SSC, 1× Blot Wash at 65°C. The mammalian species blot was washed according to the protocol provided by Clontech and hybridized and washed were carried out according to the protocol provided by Clontech.

**RESULTS**

**T81-3 Transgenic Mice Carry a Recessive Prenatal Lethal Mutation**

Transgenic line T81-3 was generated on a C57BL/6J (B6) background by the microinjection of a 10.4-kb human genomic CD\(_{8}\alpha\) transgene construct, T81. Three to five copies of the T81 transgene are present in T81-3 heterozygotes, which are phenotypically normal, but which express low levels of human CD\(_{8}\alpha\) mRNA in spleen, thymus, and brain (data not shown). Initial evidence for the presence of a recessive prenatal lethal mutation in line T81-3 came from screening progeny of intercrosses between T81-3 homozygous mice. Fifty-six live born offspring were tested for T81-3 sequences by a quantitative Southern blot analysis (data not shown); 35 (63%) were heterozygous, 21 (37%) were nontransgenic, and none were homozygous for the transgene insertion.

The absence of live born T81-3 homozygotes is not due to a deleterious effect of human CD\(_{8}\alpha\) expression. Twelve other independent lines generated with the T81 transgene construct express human CD\(_{8}\alpha\) in the same pattern and at the same levels as heterozygous T81-3 mice; yet, viable homozygous mice were obtained in each of these 12 lines (N. Lonberg and E. Lacy, unpublished results). In addition, human CD\(_{8}\alpha\) is functionally inert in the mouse, as it is unable to bind to the murine homologue of its receptor, the major histocompatibility complex (MHC) class I molecule (Kalinke et al., 1990, and Irwin et al., 1989). Thus, the absence of live born homozygous T81-3 mice indicated that the integrated transgene disrupted a developmentally essential gene and caused a recessive prenatal lethal mutation.

**T81-3 Homozygotes Become Developmentally Arrested during Gastrulation**

To determine the stage of development affected by this insertional mutation, embryos from T81-3 intercrosses were examined at different times of gestation. At midgestation most of the recovered embryos were normal, but about one-fourth of the decidua contained embryos that had been completely resorbed. Earlier, at E8.5, however, approximately one-fourth of the embryos exhibited a distinct mutant phenotype (Fig. 1). The mutant embryos were smaller than their normal littermates and the embryonic ectoderm was underdeveloped relative to the extraembryonic portion of the embryo.

Table 1 summarizes the percentages of abnormal embryos generated at E7.75, E8.5, E9.5, and E13.5 by T81-3 intercrosses and by control matings between wild-type B6 mice or non-transgenic siblings of T81-3 mice. At E13.5, 23% of the T81-3 embryos were completely resorbed, as were a small percentage (5%) of control embryos. At E9.5, abnormal embryos either exhibited the distinct mutant phenotype or were partially resorbed. At E8.5 and E7.75, abnormal embryos were classified either as exhibiting the mutant phenotype or as having been resorbed shortly after implan-
FIG. 1. Photomicrographs of E8.5 normal and mutant embryos from a T81-3 intercross mating. (Left) A normal E8.5 embryo at the early somite stage. Except for the allantois and amnion, all extraembryonic structures have been removed. (Right) An E8.5 embryo exhibiting the characteristic mutant phenotype. Only the outermost extraembryonic structure, the parietal yolk sac, has been removed; all other embryonic and extraembryonic structures were left intact. Both photomicrographs are at the same magnification.

Embryos in the latter class were found in similar percentages in T81-3 intercrosses and control matings, whereas embryos exhibiting the mutant phenotype were observed only in the T81-3 intercrosses. At all stages examined, the percentage of mutant/resorbed embryos was not significantly different from the predicted value of 25% homozygous embryos ($\chi^2$ analysis). These data are consistent with the effects of a recessive mutation that acts during gastrulation prior to E7.75.

In a series of T81-3 intercross matings separate from that summarized in Table 1, the genotypes were determined for all dissected embryos using the PCR assay described under Materials and Methods and diagrammed in Fig. 5. Five litters were collected between E7.5 and E9.5, which yielded 38 embryos. Of these, 10 (26%) exhibited the characteristic mutant phenotype shown in Fig. 1, while the rest were morphologically normal. All 10 mutants, but none of the normal embryos, were found to be homozygous for the T81 transgene insertion. Of the remaining 28 embryos, 9 (24%) were non-transgenic and 19 (50%) were heterozygous for the T81 transgene insertion.

Histological Examination of the Mutant Phenotype

The mutant phenotype was characterized in more detail by examining paraffin sections of embryos generated from T81-3 intercrosses between E6.0 and E9.5. Representative examples of these sections are shown in Figs. 2 and 3. All sections of T81-3 intercross embryos examined at a Pre Streak stage (E6.0) appeared normal (data not shown). However, between E6.5 and E7.0, the Early Streak stage, aspects of the mutant phenotype began to emerge. In normal embryos of this stage, primitive streak formation is initiated at the junction between the embryonic and extraembryonic ectoderms, an event that marks the beginning of gastrulation and that defines the posterior end of the embryonic axis. Cells delaminate through the primitive streak from the epithelial embryonic ectoderm to produce a layer of mesoderm cells that lies between the visceral endoderm and embryonic ectoderm. In both normal and presumptive mutant embryos, mesoderm formation was observed (Figs. 2A, 2B, 2D, and 2E). In the presumptive mutant embryos, however, the embryonic ectoderm appeared to have stopped growing and to be shortened relative to the extraembryonic ectoderm.

As development proceeds to the Mid Streak stage in normal embryos, the primitive streak extends anteriorly, and the newly formed mesoderm migrates anteriorly and laterally, as well as into the extraembryonic portion of the embryo. Similar streak movement was observed in the mutant embryos (Figs. 2D-2F, 3B, and 3C).

During the Mid to Late Streak stages in normal embryos,
TABLE 1

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total</th>
<th>Normal</th>
<th>Abnormal Mutant</th>
<th>Early resorptions</th>
<th>Unclassified/resorbed</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>E7.75 T81-3 52</td>
<td>35</td>
<td>15 (29%)</td>
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<tr>
<td>E8.5</td>
<td></td>
<td></td>
<td>T81-3 76</td>
<td>73</td>
<td>0 (0%)</td>
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<td></td>
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<td></td>
<td>E9.5 Control 102</td>
<td>67</td>
<td>28 (27%)</td>
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<td></td>
<td></td>
<td></td>
<td>T81-3 69</td>
<td>69</td>
<td>0 (0%)</td>
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<td></td>
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<td></td>
<td>E13.5 Control 46</td>
<td>37</td>
<td>5 (5%)</td>
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<td></td>
<td></td>
<td>T81-3 75</td>
<td>58</td>
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Note. Embryos generated from T81-3 intercrosses or control matings were examined at the indicated days of gestation. Control embryos were derived from matings between wild-type B6 mice at E13.5 and E9.5 and from matings between non-transgenic siblings of T81-3 mice at E8.5. At E8.5 and E7.75, abnormal embryos from the T81-3 intercrosses were classified either as having the characteristic mutant phenotype shown in Fig. 1 or, based on decidual size, as having been resorbed shortly after implantation. At E9.5, some abnormal embryos were nearly resorbed, while others exhibited the mutant phenotype. At E13.5, all abnormal embryos were completely resorbed. The percentage of mutant T81-3 embryos observed at E8.5 and E7.75 did not differ significantly from the predicted value of 25% homozygous embryos. The percentage of T81-3 early resorptions did not differ significantly from the percentage of control early resorptions ($\chi^2$ analysis).

The amn Mutation Maps to the Distal Region of Mouse Chromosome 12

To genetically map the amn mutation and to determine if it might be allelic to any known gene or genetic locus, we performed an interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × Mus spretus)F1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1900 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and M. spretus DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using two different probes prepared from mouse sequences flanking one of the junctions [junction 2 (Jxn2)] with the inserted T81 transgene. As shown in Fig. 5, these two probes, the 3.2- and 1.1-kb Xbal fragments, are separated by 6.1 kb at Jxn2. The 4.7-kb Xbal and 5.2-kb HindIII M. spretus RFLPs (see Materials and Methods) were used to follow the segregation of the Jxn2 sequences in the backcross mice. The mapping results indicated that the Jxn2 sequences are located in the distal region of mouse chromosome 12 linked to Aat and Ighc. Although 149 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 4A), up to 179 mice were typed for some pairs of markers. Each locus
Candidate Gene at the Mouse amn Locus

FIG. 4. amn(jxn2) maps in the distal region of mouse chromosome 12. (A) amn(jxn2) was placed on mouse chromosome 12 by interspecific backcross analysis. The segregation patterns of amn(jxn2) and flanking genes in 149 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 149 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (B6 × M. spretus)F1 parent. The shaded boxes represent the presence of a B6 allele and white boxes represent the presence of a M. spretus allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 12 linkage map showing the location of amn(jxn2) in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centiMorgans are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Database), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD). (B) Assignment by FISH analysis of the human CD8α transgene, T81, to chromosome 12 region F in the amn/+ transgenic mouse. The dots and the arrow indicate that all 16 spreads examined showed hybridization signals in region F with the CD8α probe.

was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere- Aat – 8/179 – amn(jxn2) – 4/151 – Ighc. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] are – Aat – 4.5 ± 1.5 – amn(jxn2) – 2.7 ± 1.3 – Ighc.

Occasionally random pieces of DNA, from different chromosomal locations, are found co-integrated at transgene/host DNA junctions. Therefore, to establish that the amn mutation actually maps to the same chromosomal location as the jxn2 sequences, we chromosomally mapped the integrated T81 transgene by fluorescence in situ hybridization (FISH) analysis. Metaphase chromosome spreads were prepared from lymphocyte cultures generated from an amn/+ mouse and the biotin-labeled probe was synthesized from the 10.4-kb T81 transgene. Of 16 cells examined, 16 (100%) showed signals on each chromatid of one chromosome 12 at region F, the chromosomal region predicted by the interspecific backcross analysis. Such a finding demonstrates that the 3.2- and 1.1-kb XbaI fragments do not derive from random pieces of DNA junctions.

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FIG. 5. Schematic diagram of Jxn2. XbaI fragments are shown underneath the top line; all are proportional in size except for the 5-kb fragment. The 1.1- and 3.2-kb fragments were the unique sequence probes used in the interspecific backcross analysis summarized in Fig. 4A. N, NotI site; f, transgene insertion site. PF, PR, PRT, primers used in the PCR assay to distinguish heterozygous, homozygous, and wild-type embryos; see Materials and Methods. Bottom line: exon–intron structure of the TRAFamn gene. Stippled boxes depict protein coding sequences and open boxes untranslated sequences. The arrowhead designates the location of the probe used in the RNase protection assay shown in Fig. 7. The 5' region of the TRAFamn gene lies at an unknown distance upstream from the 10-kb XbaI fragment.

DNA co-integrated with the transgene, but instead, that they represent an integral part of the amn locus that is disrupted by the transgene insertion on chromosome 12. Examination of the karyotypes revealed no chromosomal aberrations, indicating that the transgene insertion at the amn locus is not accompanied by any cytogenetically detectable deletion, insertion, or other DNA rearrangement.

We have compared our interspecific map of chromosome 12 with a composite mouse linkage map that reports the map location of many mouse mutations (provided from the Mouse Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). amn mapped in a region of the composite map that lacks mouse mutations with a phenotype similar to amn.

The distal region of mouse chromosome 12 to which amn maps has synteny with human chromosome 14q32 (summarized in Fig. 4A). This suggests that the human homolog of amn will reside on 14q32 as well.

Identification of a 7.5-kb Transcript Encoded at the amn Locus

A number of clones containing junction fragments between the T81 transgene and host flanking mouse DNA were isolated from a total genomic amn/+ kidney library using a human CD8 cDNA clone as probe. A search for amn gene candidates was initiated within these cloned junctions and our first efforts concentrated most heavily on junction 2 (Jxn2, Fig. 5), as the mouse sequences flanking this junction contain a NotI site and thus a potential Cpg island (Bird, 1987). To assess whether any of the unique sequences flanking Jxn2 contain an exon or other conserved sequence, Southern blot hybridization analyses were conducted to look for cross-species homologies. In the experiment performed with the 1.1-kb XbaI fragment, single bands were detected in human and Xenopus, as well as in mouse, DNA (data not shown and Fig. 9), suggesting that the 1.1-kb fragment contains a conserved sequence, perhaps an exon from the gene whose disruption is responsible for the amn mutation.

To determine whether the 1.1-kb fragment includes a transcribed sequence, it was used as a probe on Northern blots of total RNAs from adult tissues and embryos at different gestational stages. Figure 6 shows that the 1.1-kb probe detected a 7.5-kb transcript in both adult and embryo RNAs. In adults, the highest levels of expression were found in brain; lower levels were detected in kidney, heart, thymus, spleen, lung, and muscle (Fig. 6A). The 7.5-kb transcript was also found in testis and ovary (data not shown). No expression was detected in liver by either Northern or RNase protection analysis (Figs. 6A and 7). As shown in Fig. 6B, ES cells contained the 7.5-kb transcript. This finding suggests that the gene encoding the 7.5-kb RNA may normally be transcribed as early as the blastocyst stage of pre-implantation development. Figures 6C and 6D examine the expression of the 7.5-kb transcript during postimplantation stages of development. This transcript was found at E7.5, during gastrulation, and the Northern analysis in Fig. 6C indicates that the 7.5-kb transcript is present at higher levels on E6.5 than on E7.5. Thus, the 7-kb transcript is normally expressed during early embryogenesis and, in particular, at stages of gastrulation when the amn mutant begins to exhibit phenotypic defects (E6.5–E7.5). In situ hybridization analyses to sections of normal E6.5 and E7.0 embryos show that the 7.5-kb transcript is ubiquitously expressed throughout the embryonic and extraembryonic regions of the gastrulating embryo (Fig. 6E and data not
FIG. 6. Northern and in situ hybridization analyses of the 7.5-kb transcript. (A–D) Northern analysis using as probe the conserved 1.1-kb XbaI fragment from Jxn2. (A) Total RNAs from B6 adult tissues, 20 μg per lane. (B) Total RNA prepared from ES cell cultures. ES, embryonic stem cells purified away from embryonic fibroblasts (EFs) by plating trypsinized cultures for 20 min and then collecting the supernatant; less than 1% of the recovered cells are contaminating EFs. EF, embryonic fibroblasts cultured in the absence of ES cells. ES + EF, trypsinized ES cultures before the plating step to purify ES cells; 1–10% of the recovered cells are contaminating EFs. The ES + EF lane contains approximately 1.5× the amount of RNA loaded in the EF and ES lanes. (If the signal in the ES + EF lane derived solely from the contaminating EFs, then the signal in the ES lane should be much less intense than that in the ES + EF lane, rather than of roughly equal intensity as observed.) (C) Total RNAs prepared from normal E6.5 (2 μg) and E7.5 (7 μg) embryos. (D) Total RNAs (20 μg) prepared from normal embryos at different stages of gestation; independent preps on the same gestational day are designated a or b. The probe was a radiolabeled T7 transcript of the 1.1-kb XbaI fragment from Jxn2. (E) In situ hybridization analysis using radiolabeled antisense and sense transcripts of the TRAFamn open reading frame as probes. Panels i–iv depict parasagittal sections of a wild-type E7.0 Mid Streak B6 embryo. The antisense and sense sections are adjacent to each other. i, iii: bright field; ii, iv: dark field. The photographs were taken by an Axiophot microscope under 20× magnification.
The 7.5-kb Transcript Encodes a Protein with Homology to TRAFs, Tumor Necrosis Factor Receptor-Associated Factors

In three successive steps 7.3 kb of the 7.5-kb transcript were cloned from three independent mouse brain cDNA libraries. Seven independent cDNA clones terminate at the same 3' site with a string of As that begins 11 bases downstream from an AAUAAA motif; therefore the composite 7.3-kb cDNA contains the entire 3' untranslated region. The difference in size between the 7.3-kb composite cDNA and the 7.5-kb transcript most likely resides largely in the poly(A) tail contained in the transcript. Thus, the 7.3-kb cDNA probably represents a nearly full-length clone, with any missing sequence deriving from the 5' untranslated region. The nucleotide sequence of the composite cDNA includes an open reading frame of 567 amino acids, a 3' untranslated region of approximately 5.0 kb, and a 5' untranslated region of about 0.64 kb.

The initial search of the GenBank database revealed that the protein encoded by the 7.5-kb mRNA shares significant homology with the mouse TNF receptor-associated Factors 1 and 2 (TRAF1 and TRAF2). TRAF1 and TRAF2 define a new family of putative signal transducing proteins that associate with the cytoplasmic domain of TNF receptor 2 (TNF-R2), a receptor involved in stimulating the proliferation of T cells and mononuclear cells in response to TNF.
Candidate Gene at the Mouse amn Locus

FIG. 8. Schematic diagram of the TRAFamn message and encoded protein. The protein domains are designated based on the analysis of Cheng et al. (1995). AA, amino acid identity between human and mouse of the five protein domains; NT, nucleotide sequence homology of the different domains and of the 3'-UTR1 (untranslated region). 3'-UTR2 (approximately 4.5 kb) is not present in the human message. The arrowheads represent the locations of known introns.

(Rothe et al., 1994). TRAF2 can bind directly to TNF-R2, whereas TRAF1 appears to associate with TNF-R2 by binding to TRAF2 through a COOH terminal homology region, termed the TRAF domain (Rothe et al., 1994). Because the protein encoded by the 7.5-kb transcript shares 59% identity with TRAF1 and 62% identity with TRAF2 in the TRAF domain, we named the gene encoding this protein TRAFamn, for TRAF at the amn locus. TRAF-C domain. One difference between the human and mouse messages is that the former is 2.5 kb in length, while the latter is 7.5 kb. Most of this difference in size is accounted for by about 4.5 kb of 3' untranslated sequence that is present only in the mouse transcript.

More recent publications indicate that TRAFamn is identical to a protein that binds the cytoplasmic domain of CD40, a member of the TNF receptor superfamily involved in regulating the survival, proliferation, and differentiation of B cells. Two groups, which cloned the human gene, named this protein CD40 binding protein (CD40bp; Hu et al., 1994) and CD40-associated protein-1 (CAP-1; Sato et al., 1995), whereas another group, which cloned both the human and mouse genes, named it CRAF1 (Cheng et al., 1995). Interestingly, TRAFamn has been cloned a fifth time in experiments to identify cellular proteins that bind the cytoplasmic domain of Epstein-Barr Virus (EBV) transforming protein, LMP1 (Mosialos et al., 1995). In this case, the TRAFamn protein has been named LAP1, for LMP1-associated protein.

The TRAFamn Gene Is Evolutionarily Conserved in both Mammalian and Nonmammalian Species

As reported by Cheng et al. (1995), mouse and human TRAFamn/CRAF1 share 96% overall amino acid identity and encode a structurally complex protein. Their analysis predicts that the TRAFamn/CRAF1 protein contains five distinct structural domains: a Zn ring finger, a stretch of five Zn fingers, an isoleucine zipper, TRAF-N, and TRAF-C. The TRAF-C domain is sufficient for binding to the CD40 receptor and for TRAFamn/CRAF1 homodimerization (Cheng et al., 1995; Sato et al., 1995). TRAF-N encodes a putative coiled-coil. Figure 8 lists the amino acid identities between human and mouse for each of these domains. The TRAF-C and isoleucine zipper domains share 100% amino acid identity, whereas the Zn ring finger, Zn finger, and TRAF-N domains are only slightly less conserved with 95.2-96.1% amino acid identities.

Our data demonstrate that the nucleotide sequences of the human and mouse TRAFamn/CRAF1 messages are also highly conserved. The cDNA sequences share 84.5% overall nucleotide homology and this homology extends into the 3' untranslated region (Fig. 8). In fact, the 1.1-kb XbaI probe that initially detected cross-species homology and identified the 7.5-kb transcript, derives almost entirely from the 3' untranslated region; the only protein coding region present in the 1.1-kb XbaI fragment is the last 78 bp of the TRAF-C domain. One difference between the human and mouse messages is that the former is 2.5 kb in length, while the latter is 7.5 kb. Most of this difference in size is accounted for by about 4.5 kb of 3' untranslated sequence that is present only in the mouse transcript.

As indicated in Fig. 8, among the five protein domains, TRAF-C exhibits the highest nucleotide sequence homology between the mouse and human genes. Consequently, to evaluate the evolutionary conservation of the TRAFamn gene across a wider spectrum of species, zoo blot analyses were performed using the TRAF-C domain as a probe. Cross-hybridizing bands were observed in all mammalian species tested including, besides human and mouse, monkey, rat, dog, and cow. In addition, cross-hybridizing bands were detected in chicken and snake, the only two nonmammalian vertebrates assayed to date with the TRAF-C probe. The same patterns of cross-species hybridization were obtained using the 1.1-kb XbaI fragment as a probe (Fig. 9).

FIG. 9. Zoo blot analysis with the 1.1-kb XbaI probe. (A) Southern blot, purchased from Clontech, contains 8 \( \mu \)g of EcoRI-digested genomic DNA in each lane. (B) Southern blot contains 10 \( \mu \)g of EcoRI-digested genomic DNA in each lane. Both blots were probed with the 1.1-kb XbaI fragment from the 3' untranslated region of TRAFamn. Note that the upper band in the mouse lane in A may represent a TRAFamn family member.
DISCUSSION

amn Is a Recessive Gastrulation Stage Mutation

We have characterized a new recessive gastrulation stage mouse mutation, amnionless. This mutation was generated by the integration of three to five copies of a human CD8 transgene into the distal region of mouse chromosome 12. Histological analyses of the embryos produced from amn/+ intercrosses demonstrate that the amn mutant first appears defective at the Early Streak stage, between E6.5 and E7.0, and that it dies and is resorbed between E9.5 and E10.5.

The phenotypic studies presented in Figs. 1-3 show that the amn mutant initiates mesoderm production, but then becomes developmentally arrested between the Mid and Late Streak stages of gastrulation. While extraembryonic structures, including the chorion, yolk sac blood islands, and allantois appear to develop normally, the small embryonic ectoderm remains undifferentiated and generates no amnion. In addition, the embryonic mesoderm that is generated does not become organized into node, notochord, and somites and there is no morphological evidence of neural induction. Evaluation of these phenotypic defects in light of the fate maps of Pre and Early Streak embryos (Lawson et al., 1991; Beddington, 1994; Sulik et al., 1994; Quinlan et al., 1995) suggests that the mutated amn gene leaves proximal streak functions intact, thus accounting for the generation of morphologically normal extraembryonic tissues. However, one extraembryonic structure that fails to form normally in the amn mutant is the amnion. The amnion is a two cell layer structure; one layer derives from embryonic ectoderm and the other from extraembryonic mesoderm cells specified by the proximal primitive streak (Gardner, 1983; Lawson et al., 1991). Our analyses to date cannot determine whether the absence of the amnion results from embryonic ectoderm and/or proximal mesoderm defects. Nonetheless, the presence of other proximal streak generated structures suggests that the amn mutation may specifically affect the ectoderm precursors of amnion cells. The mutated amn gene also appears to severely perturb the functions of the distal streak and node that are required for the differentiation and patterning of embryonic mesoderm and for neural induction. We are in the process of testing these predictions by performing in situ hybridizations and immunohistochemical analyses on amn mutant embryos to examine genes whose expression patterns serve as molecular markers for distinct subsets of ectodermal and mesodermal cells in the gastrulating mouse embryo (Conlon et al., 1994; Faust et al., 1995).

amn Is a Member of a Small Group of Mutations That Appear to Specifically Perturb the Distal Streak and Embryonic Ectoderm

Of the pool of recently described gastrulation stage mutants, amn most closely resembles eed (embryonic ectoderm development, Faust et al., 1995) and Hdh (Huntington's disease gene homolog deficiency, Nasir et al., 1995; Duyao et al., 1995; Zeltlin et al., 1995). Like amn, eed and Hdh mutants initiate mesoderm production, support the development of extraembryonic structures, but fail to organize embryonic mesoderm into node, notochord, and somites. Unlike amn, however, eed and Hdh embryos can generate a two cell layer amnion. Thus, in all three mutants, proximal streak functions appear largely intact, whereas distal streak functions and embryonic ectoderm development are perturbed prior to the appearance of the neural plate. Faust et al. (1995) have hypothesized that the distal streak defects of eed may be a byproduct of a dominant posteriorization of the embryo, reflected in the ectopic expression of T and Eva1 and in the relative overproduction of extraembryonic mesoderm. A similar overproduction of extraembryonic mesoderm is not observed in amn and Hdh mutant embryos. Interestingly, the tw5 complementation group of recessive t-alleles also appears to fall into this class of gastrulation stage mutants, which exhibit distal streak and embryonic ectoderm defects prior to E7.5 (Bennett, 1975; Bennett and Dunn, 1958). Only further studies can address the important question of whether these mutations affect the same, different, or interacting pathways in the gastrulating mouse embryo.

Another feature that amn shares with eed, Hdh, and tw5 is growth retardation. This growth defect is readily apparent in amn mutant embryos by E7.5 at the Mid to Late Streak stage and may first manifest itself in the embryonic ectoderm at the Early Streak stage, E6.5. (Fig. 2D). At this time, the embryonic ectoderm appears somewhat shortened relative to the extraembryonic ectoderm in the presumptive mutants.

Growth retardation is a characteristic that has been noted in many gastrulation stage mutants besides amn, eed, Hdh, and tw5. Examples include the tw18/tw18 embryos studied by Snow and Bennett (1978), the mutant embryos generated by targeted null mutations in Fgfr-1 (Yamaguchi et al., 1994; Deng et al., 1994), HNF-4 (Chen et al., 1994), and HNF-3β (Ang and Rossant, 1994; Weinstein et al., 1994), and the irradiation induced mutant, rump white (Rw, Bucan et al., 1995). The observation that virtually all gastrulation stage mutants exhibit growth defects argues that signals promoting differentiation during gastrulation may be inextricably linked to those coordinating cell survival, growth, and proliferation.

TRAFamn Is a Candidate for the Mutated amn Gene

The TRAFamn gene fulfills three expectations of a candidate for the mutated gene that is responsible for the gastrulation stage defects of the amn mutant. First, it maps at a junction between the T81 transgene insertion and host chromosomal 12 sequences, Jxn2. Second, it is transcribed during gastrulation, and third, its expression is disrupted in amn mutant embryos. As illustrated in Fig. 5, transcription of the TRAFamn gene proceeds 5' to 3' toward the transgene insertion and terminates approximately 9 kb upstream from the transgene breakpoint. Thus, the mRNA encoding region...
of the TRAFamn gene is not physically disrupted by the transgene insertion that generated Jxn2. Therefore, the T81 insertion at this junction may have knocked out TRAFamn expression by separating the gene from essential 3' regulatory elements. However, Southern blot hybridization and genetic analyses indicate that the amn locus consists of two very tightly linked transgene insertions and, as yet, we cannot rule out the possibility that the second transgene insertion occurred in the 5' region of the TRAFamn gene. Our preliminary exon mapping experiments indicate that the last five exons of the TRAFamn gene are separated from upstream exons by an intron that is greater than 26 kb in length and that the TRAFamn gene probably encompasses more than 50 kb of chromosomal DNA. Experiments are now in progress to clone the 5' region of the TRAFamn gene to determine if it might be disrupted by the second transgene insertion.

As in the case of any transgene induced mutation, the T81 transgene insertion at the amn locus may have disrupted the expression of other genes in addition to TRAFamn. Therefore, to unambiguously establish whether the loss of TRAFamn expression is responsible for the mutant phenotype of amn embryos, we are currently generating independent null mutations in the TRAFamn gene by gene targeting in ES cells.

TRAFamn Is Identical to a Protein That Can Bind to the Cytoplasmic Domains of CD40, LMP1, and Other Members of the TNF Receptor Superfamily

The finding that a TRAF related gene is a candidate for the amn gene is intriguing. The first TRAF family members, TRAF1 and TRAF2, were identified in a screen for proteins that can associate with the cytoplasmic domain of the p75-p80 TNF receptor, TNF-R2 (Rothe et al., 1994). TNF-R2 is one of the founding members of the tumor necrosis factor receptor superfamily, which also includes TNF-R1, nerve growth factor receptor (NGFR), Fas, CD40, CD27, CD30, OX40, and 4-1BB (reviewed in Armitage, 1994; Smith et al., 1994; Beutler and van Huffel, 1994). Molecules in this receptor superfamily are defined by the presence of multiple cysteine-rich repeats of about 40 amino acids in their extracellular regions. Their cytoplasmic domains are generally small and, with the exception of TNF-R1 and Fas, lack homology among the family members. Ligands for most of the known TNF receptor superfamily members have been identified; they are type II transmembrane proteins that share sequence homology with TNFα and TNFβ in their COOH-terminal, receptor binding regions. Many distinct and overlapping functions have been defined for the known members of the TNF receptor superfamily, but in general, this receptor superfamily is involved in coordinating the survival, proliferation, and differentiation of cells in the lymphoid and hematopoietic lineages.

The cytoplasmic domains of the TNF receptor superfamily members encode no inherent enzymatic activity and thus it is not known how these receptors couple to downstream signaling pathways upon activation by ligand. TRAF1 and TRAF2 were discovered in experiments by Rothe et al. (1994) to elucidate the mechanisms by which TNF-R2 initiates signaling. These investigators found that the ubiquitously expressed protein, TRAF2, can bind directly to the cytoplasmic domain of TNF-R2, whereas the tissue-specifically expressed protein, TRAF1, can associate with TNF-R2 only indirectly by heterodimerizing with the COOH-terminal region of TRAF2. Three other groups have carried out parallel studies to identify protein factors that associate with the cytoplasmic domain of another TNF receptor superfamily member, CD40 (Hu et al., 1994; Sato et al., 1995; Cheng et al., 1995). Using the yeast two-hybrid system and B cell cDNA libraries, all three groups cloned the same gene. The protein encoded by this gene has been named the CD40 binding protein (CD40bp) by Hu et al. (1994), CAP-1 by Sato et al. (1995), CRAF1 by Cheng et al. (1995), and most recently TRAF3 by Rothe et al. (1995). Our nucleotide sequencing analysis indicates that the TRAFamn gene encodes the same protein identified by these investigators.

TRAFamn is believed to play a central role in signaling by CD40. Via the TRAF-C domain, it binds to a region of the CD40 cytoplasmic domain that is required for signaling (Cheng et al., 1995; Hu et al., 1994; Sato et al., 1995) and overexpression of the TRAF domain of TRAFamn (CRAF1) inhibited the CD40-mediated upregulation of CD23 (Cheng et al., 1995). CD40 is normally expressed on B cells, as well as on dendritic cells, follicular dendritic cells, hematopoietic progenitor cells, and epithelial cells (Banchereau et al., 1994). However, its function is best understood on B cells. The interaction of CD40 on B cells with its ligand, CD40L, on activated T cells, triggers B cells to survive, proliferate, and differentiate. Strikingly, the phenotype of the amn mutant suggests that it is deficient in promoting the survival, proliferation, and differentiation of a particular group of cells, the embryonic ectoderm cells of the mouse gastrula. This observation raises the question of whether TRAFamn, which we have shown to be expressed during gastrulation in wild-type but not in amn mutant embryos, participates in the coordination of embryonic ectoderm cell growth, proliferation, and differentiation in the mouse embryo via CD40.

Germ line knockouts of both CD40 and CD40L have been generated. In each case, viable homozygous mice are produced and they are apparently only deficient in B cell responses; in particular, these mice can not form germinal centers and their B cells cannot undergo isotype switching (Xu et al., 1994; Kawabe et al., 1994). Therefore, if TRAFamn is, in fact, essential for mouse gastrulation, then its role is not solely dependent on its association with CD40. Either complexes between TRAFamn and other members of the TNF receptor superfamily can functionally replace those between CD40 and TRAFamn in the gastrulating embryo or CD40 may not be expressed in the gastrula and TRAFamn may function exclusively through a different member of the TNF receptor superfamily.

Consistent with this argument are two recent findings showing that TRAFs can interact with multiple receptors.
First, TRAFamn has been cloned a fifth time in experiments to identify cellular proteins that bind the cytoplasmic domain of the Epstein–Barr Virus (EBV) transforming protein, LMP1 (Mosialos et al., 1995). In this case, the TRAFamn protein has been named LAP1. These studies show, by co-immunoprecipitation, that TRAFamn/LAP1 can associate with other members of the TNF receptor superfamily besides CD40. In particular, TRAFamn/LAP1 associated with the lymphotoxin β receptor (LTβR) as efficiently as it did to CD40; a weaker association between TRAFamn/LAP1 and TNF-R1 and TNF-R2 was also detected in these studies. Second, studies reported from Goeddel’s group demonstrated that TRAF2 can functionally mediate the activation of NF-κB by three different receptors: TNF-R2, CD40 (Rothe et al., 1995), and TNF-R1 (Hsu et al., 1996). Thus, it is certainly conceivable that TRAFamn could function in both lymphoid tissues and early embryos through receptor interactions that are stage and/or cell type specific. Future studies investigating the expression of TNF and TNF receptor superfamily members in the early embryo, combined with gene targeting experiments to generate an independent null mutation in the TRAFamn gene, will begin to elucidate the role this receptor family plays in coordinating the survival, proliferation, and differentiation of cells in the gastrulating mouse embryo.

The complex structure of the protein encoded by the TRAFamn gene also suggests that it could serve multiple signaling roles in a cell type- or stage-specific manner. Cheng et al. (1995) identified five distinct domains: a Zn ring-finger, a stretch of five Zn fingers, an isoleucine zipper, TRAF-N, and TRAF-C. The TRAF-C domain is sufficient for CD40 binding and homodimerization in the yeast two-hybrid system (Cheng et al., 1995; Sato et al., 1995). Based on the amino acid identity exhibited by TRAFamn to the TRAF-C domains of TRAF1 and TRAF2, 59 and 62% respectively, TRAFamn represents the third known member of the TRAF family (Cheng et al., 1995; Hu et al., 1994; Sato et al., 1995). TRAFamn is more highly related to TRAF2 than TRAF1. Like TRAFamn, TRAF2 contains Zn ring and Zn finger domains and neither of these motifs is present in TRAF1 (Cheng et al., 1995). However, neither TRAF1 nor TRAF2 contain an isoleucine zipper, whereas TRAFamn does (Cheng et al., 1995). While TRAF-N, which encodes a putative coiled-coil, and the isoleucine zipper represent structures that mediate protein–protein interactions, the Zn ring and Zn fingers are structural features that are characteristic of nuclear proteins involved in the recognition of nucleic acids (Barlow et al., 1994). These findings have led to the suggestion that TRAFamn could directly transduce signals to the nucleus (Cheng et al., 1995). These findings also raise the possibility that TRAFamn participates in a number of different cellular processes by interacting with different, and potentially cell type-specific, proteins or complexes via its distinct domains. In this way, TRAFamn could participate in the regulation of cell survival, proliferation, and differentiation in both the early embryo and B cells.

TRAFamn is highly conserved between human and mouse, exhibiting 96% overall amino acid identity (Cheng et al., 1995). Our zoo blot analyses show that the TRAFamn gene is also highly conserved in all of the mammalian and nonmammalian vertebrates we have tested. Such a high degree of evolutionary conservation suggests that TRAFamn may play a fundamental role in eucaryotic cell survival and proliferation, and if this is true, we would predict that TRAFamn would be conserved in nonvertebrates as well. Interestingly, the first 395 nt of the mouse TRAFamn 3′ untranslated region possess 76.7% homology with the human 3′ untranslated region. The high conservation of this noncoding region between human and mouse, and potentially between mouse and other mammalian and nonmammalian vertebrates as well (Fig. 9), raises the question of whether it plays a critical role in regulating the time, level, and/or location of TRAFamn expression.

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