Molecular cloning of Notch, a locus affecting neurogenesis in Drosophila melanogaster

gene isolation/gene localization

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ABSTRACT The Notch locus is one of the best characterized loci in Drosophila melanogaster in terms of its genetic structure and developmental effects. Mutations in this locus profoundly affect the differentiation of the early embryo. Using an inversion involving the Notch locus and previously cloned sequences, we have isolated chromosomal segments from the Notch region (3C7) encompassing 80 kilobases (kb) of DNA. Based on comparison between mutant and wild-type DNA, we have positioned cloned sequences within the Notch genetic map; furthermore, we have defined a region of approximately 40 kb within which the structural lesions correlating with all Notch alleles mapped to date appear to reside. We have examined the transcriptional activity of the cloned sequences during ontogeny and find a single size class of poly(A)+ RNA, 10.5 kb long, that is homologous to sequences within this 40-kb region. We conclude that DNA sequences belonging to the Notch locus have been cloned and that the 10.5-kb poly(A)+ RNA is essential for wild-type Notch function. We discuss these structural and transcriptional data in light of the existing genetic and developmental characterization of the Notch locus.

The first visible signs of ectodermal differentiation in Drosophila melanogaster appear approximately 4 hr after fertilization. At this time, the precursor cells of the central nervous system segregate from the apparently homogeneous ectodermal germ layer. The neuroblasts appear to arise from, and are confined to, what is termed the neurogenic region (1). Very little is known about how the neurogenic region is defined or about the factors that direct the determination and differentiation of the ectoderm. Even so, the importance of the genetic control on ectodermal differentiation was noted long ago by Poulson (2), who observed that deficiencies involving the Notch locus led to abnormal embryonic development. An embryo homozygous for a Notch deficiency exhibits hypertrophy of the nervous system at the expense of hypodermal structures, as if a switch in ectodermal determination is affected. The classic studies of Poulson have been confirmed and extended by Campos-Ortega et al. (3). Six other loci have been identified that can produce an early embryonic phenotype similar to that associated with Notch (ref. 3; C. Nusslein-Volhard, E. Weischaus, and H. Kluding, personal communication).

To gain a deeper insight into the events leading to ectodermal differentiation, we have initiated a study directed toward the molecular characterization of these loci. We have begun with the analysis of Notch, which is best understood, genetically and phenotypically (4, 5). The Notch locus, symbolized N, is located at band 3C7 of the salivary gland chromosomes and is genetically defined by an array of mutations that, when heterozygous, yield a dominant phenotype consisting of variably notched wings, thickened wing veins, and minor bristle abnormalities (6). N mutations are also recessive lethals since homozygous or hemizygous animals die as embryos, displaying a hypertrophied nervous system. Two additional classes of mutation have been shown to be allelic to these lethal mutations. The first class is a group of recessive visibles that affect either wing or eye morphology (7). These fall into three complementation groups, facet (fa), split (spl), and notchoid (nd), members of which will complement each other but fail to be complemented by N alleles. The second class comprises the dominant Abruptax (Ax) mutations, which affect wing veination and exhibit complex interactions with the N alleles (8, 9). The embryonic lethality associated with Notch suggests a requirement for the gene product(s) during embryogenesis. Moreover, the phenotypes associated with constitutive and conditional mutations within Notch indicate a requirement for temporal and spatial regulation of Notch expression during later development (10). In spite of the detailed genetic and embryological characterization of this locus, however, the biochemical nature and the mode of action of its product(s) remain unclear (11).

MATERIALS AND METHODS

Embryonic DNA (12), a phage DNA (12), and cosmid DNA (13) were isolated as described in the indicated references. DNA from Drosophila adults was isolated by an unpublished method developed by R. Lifton (Stanford University), with minor modifications. RNA was prepared as described in Fig. 4.

Electrophoresis of restriction enzyme-cleaved DNA and preparation of DNA blots onto nitrocellulose were carried out according to standard procedures (14). RNA was fractionated on agarose gels containing formaldehyde and blots were prepared with minor modifications as described by Maniatis et al. (14). Conditions for hybridization, autoradiography, and nick-translation are also described in ref. 14. Detection of Drosophila repetitive sequences in recombinant molecules was achieved using "reverse" Southern blot analysis, by hybridizing 0.02–0.1 μg (approximately 5–10 × 10⁶ cpn of [3²P] of nick-translated genomic Oregon R DNA to nitrocellulose filters containing 1 μg of cloned DNA that had been cleaved with restriction enzymes, electrophoretically fractionated, and transferred to nitrocellulose. Hybridization was conducted overnight under the conditions indicated above.

RESULTS

Molecular Definition of a Notch Chromosomal Rearrangement. Our approach to cloning the Notch locus consisted of isolating the chromosomal region, 3C7, in which Notch

Abbreviations: kb, kilobase(s) or kilobase pair(s); cM, centimorgan(s).
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is known to reside. This could be accomplished by the successive isolation of overlapping DNA segments beginning with a unique cloned sequence containing the salivary glue secretion protein gene sgs4, which has been mapped to region 3C11-12 (12). The extent of this chromosomal "walk" was substantially reduced by the molecular definition of the mutation N7688, a chromosomal inversion between 3C7 and 3C11-12 (13).

A 3-kilobase (kb) HindIII fragment (henceforth the 3-kb probe) located approximately 6.5-kb distal to the sgs4 gene was hybridized to blots of EcoRI-digested DNAs isolated from wild-type and w&u03b6;7068 Y; Dp(1;2) 51b7/+ flies. While both samples exhibit a homologous fragment 7-kb long, the DNA isolated from the flies containing the mutation also exhibits additional fragments 6.8 and 2.6 kb long (data not shown). The 7-kb fragment reflects wild-type organization and is detected in the mutant DNA sample because of the presence of an insertional translocation, white+51b7, required to cover the lethality associated with the Notch mutation (15). We infer that the additional EcoRI fragments (6.8 and 2.6 kb) evident in the mutant reflect the presence of the N7688 inversion breakpoint in the vicinity of sgs4. The possibility that the additional fragments arise as the result of restriction site heterogeneity is eliminated by the fact that the parental chromosome used for the generation of N7688, fa+w63 (15), exhibits wild-type organization in this region.

Cloning Sequences from the Notch Locus. Isolation of the N7688 fragments complementary to the 3-kb probe, and presumably containing Notch sequences, began with the construction of a hybrid phage library (14) using λ 607 (16) as a vector and EcoRI-digested DNA isolated from w&u03b6;7068 Y; Dp(1;2) 51b7/+ adult flies. Two groups of recombinants homologous to the 3-kb probe could be defined on the basis of their restriction enzyme cleavage pattern. The first group, comprising four phage, contained a 7-kb EcoRI DNA segment having an organization indistinguishable from that of the corresponding wild-type 3C11-12 region. These phage were derived from the duplication Dp(1.2) +51b7. The second group contained EcoRI inserts of either 6.8 kb (one phage) or 2.6 kb (two phages) corresponding to the N7688 breakpoint fragments defined by the analysis discussed above.

Twenty recombinants were identified by using the 6.8-kb breakpoint fragment as a hybridization probe to screen a Canton S phage library (14). Seven phage failed to exhibit homology to the 3-kb probe derived from the 3C11-12 region. Restriction enzyme analysis of these phage yielded approximately 25 kb of contiguous sequence. The sequence organization of this interval bears no resemblance to the 3C11-12 region, suggesting that the newly cloned region spans the 3C7 breakpoint of N7688.

The left lane of Fig. 1B shows a DNA blot of wild-type DNA digested with EcoRI and probed with λ cDM 2941, a phage derived from the newly cloned region that contains the N7688 breakpoint. In situ hybridization of λ cDM to wild-type chromosomes (Fig. 1A) defines a single site of hybridization, which we identify as 3C7. Hybridization of both the 6.8- and 2.6-kb N7688 EcoRI breakpoint fragments to EcoRI-digested wild-type chromosomal DNA reveals homology to a 2.2-kb fragment in addition to the expected 7-kb fragment derived from the 3C11-12 region (data not shown). The fact that the two pairs of fragments (6.8 kb plus 2.6 kb and 2.2 kb plus 7.0 kb) sum to the same length, within experimental error, supports the contention that N7688 is a simple inversion.

The work reported here was begun after the feasibility of this approach as a means of gene isolation had been demonstrated by W. Bender, P. Spierer, and D. S. Hogness.
each cloned segment was obtained by comparative Southern blot analysis of recombinants and Oregon R genomic DNA. Southern blot analysis of genomic DNA also provided a means by which repetitive sequences could be identified within the cloned region. In addition, we routinely tested for the presence of repetitive sequences by reverse Southern blot analysis.

Within the 80-kb cloned region, we have localized repetitive sequences at two sites. A repetitive sequence that has not been characterized in detail is found between coordinates +8 and +9.5 in both Canton S and Oregon R. In contrast to this sequence, another repetitive element, approximately 6 kb long, is found only in Canton S (Fig. 2), suggesting that this insertion may represent a mobile genetic element (19). The remainder of the cloned sequences appear to be unique on the basis of both standard and reverse Southern blot analysis.

Correlation Between the Physical and the Genetic Maps.

Molecular lesions corresponding to specific mutations that have been localized by recombination on the Notch genetic map should fall in an array along the physical map as predicted by their respective genetic map positions. We sought to establish the correlation between molecular alterations and known genetic lesions within the locus by comparative Southern blot analysis of mutant and wild-type DNAs.

The lesions most readily definable in molecular terms are those involving gross rearrangements—that is, those that visibly alter normal cytology. The nature of physical alterations predicted on the basis of Southern blot analysis can be confirmed in such a mutant by in situ hybridization of appropriate cloned segments to the mutant chromosome. N7531 is a small deficiency between 3C7 and 3C10-12, the breakpoint of which has been mapped 0.038 centimorgan (cM) proximal to N560 (Fig. 3C). In situ hybridization of a cDNA 2930 (Fig. 2) to a N7531 chromosome detects homology to the 3C7 region;
in contrast, \(\lambda\) cDm 2941 (Fig. 2) shows no homology to 3C7. This is consistent with the localization of the deficiency breakpoint between coordinates \(-20\) and \(-10\) by Southern blot analysis (Fig. 3). The molecular lesion associated with inversion \(N^{668}\) described above maps between \(-1.1\) and \(+1.1\), to the right of that associated with \(N^{7631}\). This would be anticipated since the genetic map position of the distal breakpoint of \(N^{668}\) is 0.122 cM proximal to \(N^{7631}\).

A second group of mutations for which molecular alterations can be reliably defined is comprised of those for which the parental strains are known. The belief that a given molecular alteration is the cause of a specific mutant phenotype is considerably strengthened if we can eliminate the possibility that the same alteration was present in the wild-type parent. The \(fa^2\) mutation, which maps between \(fa\) and \(fa^3\), arose spontaneously on a \(w^{nd}\) background (W. J. Welshons, personal communication). Comparison of the mutant and parent chromosomes reveals an alteration between coordinates \(-13\) and \(-10\). With the problem of isogenicity in mind, we have generated a set of dominant Notch alleles by x-ray mutagenesis on a single parental background. Comparative Southern blot analysis of three of these mutants (see also Discussion), \(N^{6166}\), \(N^{6114}\), and \(N^{6116}\), and their Oregon R parent has allowed us to define alterations within specific restriction fragments indicated in Fig. 3.

On the basis of the data available for \(N^{7631}\), \(fa^3\), and \(N^{668}\), we can formulate a relationship between physical and genetic maps of the Notch locus. If this relationship is accurate we should be able to predict the physical location of mutations that have been mapped within the locus by recombination. In fact, three such mutations: \(N^{668}\), \(N^{664-40}\), and \(N^{662}\), exhibit restriction enzyme cleavage pattern alterations consistent with their respective genetic map positions (Fig. 3).

**Transcriptional Activity of the Notch Locus.** We have investigated the transcriptional activity of the cloned 3C7 region by hybridizing a series of radioactively labeled DNA fragments spanning 80 kb (coordinates \(-40\) to \(+40\)) to blots of agarose gels containing electrophoretically fractionated RNAs isolated from various developmental stages (Fig. 3). These experiments allowed us to define discrete size classes of RNA, 10.5 kb, 7 kb, 0.7 kb, and 0.9 kb and a family of transcripts ranging from 3 to 10 kb in length. (The sizes given are the best estimates available to date and are of limited (approximately 10%) precision.) With two exceptions, fragments mapping between approximately \(-29\) and \(+12\) exhibit homology to the 10.5-kb RNA. The 2-kb EcoRI fragment between \(+9.5\) and \(+11.5\) is homologous to two additional size classes of RNA, 0.9 kb and 0.7 kb. These RNAs are also detected with the 3.3-kb EcoRI fragment mapping between \(+13.9\) and \(+17.2\). The 1.55-kb EcoRI fragment shown to contain a repetitive element exhibits homology to transcripts ranging in length from 3 to 10 kb. The 5-kb XhoI fragment (\(-27\) and \(-32\)) detects transcripts 7 kb long.

Our attention is drawn in particular to the 10.5-kb transcript. Accumulation of this RNA is developmentally regulated, as shown by the low-resolution developmental profile (Fig. 4). As mentioned above, we find that all mutations thus far mapped within the Notch locus fall within a region approximately 40 kb long located roughly between coordinates \(-30\) and \(+10\). Interestingly, fragments derived from the same 40-kb interval exhibit homology to a discrete size class (10.5 kb) of RNA. At the level of resolution of the analysis shown in Fig. 3, we can define weakly hybridizing areas as well as one clear discontinuity in hybridization (\(-1.1\) to \(+1.1\)) within the 10-kb region. It is therefore reasonable to suggest that the 10.5-kb RNA is the mature processing product of a much larger primary transcript that spans the 40-kb region. The relationship, if any, between the 10.5-kb RNA and the flanking transcripts, 0.7 kb, 0.9 kb, and 7 kb long, or the family of transcripts 3-10 kb long is not known. Moreover, it should be emphasized that based on these data we cannot exclude the possibility that the 40-kb region encodes more than a single 10.5-kb transcript, nor can we be certain that homology to 10.5-kb RNA does not extend beyond the 80-kb region depicted in Fig. 3.

**DISCUSSION**

Our belief that the cloned interval characterized in this report contains Notch locus sequences rests principally on a correlation between structural alterations and specific mutations and is supported by the transcriptional activity of these sequences during development.

The attempt to correlate physical lesions and Notch mutations within the cloned region is hampered by the chromosomal DNA heterogeneity observed among strains of *Drosophila* (see, e.g., figure 3 of ref. 19). Therefore, a minimum requirement for the identification of a specific molecular lesion as the cause of a Notch mutation is knowledge of the chromosomal background on which the mutation was induced. Though a large number of mutations mapping within Notch have been isolated, this requirement is met for very few. Among the recessive visible alleles only in one case, \(fa^3\), is the parent chromosome available. Apart from \(N^{668}\), among the dominant Notch alleles depicted in Fig. 3, three (\(N^{6166}\), 

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**Fig. 4.** Developmental profile for the 10.5-kb RNA. Poly(A)\(^+\) RNA isolated from animals at various developmental stages was electrophoretically fractionated on agarose gels, transferred to nitrocellulose, and hybridized with a \(32^P\)-labeled 7-kb fragment mapping between coordinates \(+1.1\) and \(+8.1\) (Fig. 3). RNA was prepared from appropriately staged Oregon R animals by homogenization in a 1:1 mixture of extraction buffer (50 mm Tris-HCl, pH 7.5/0.9% NaDodSO\(_4\)/100 mm NaCl) and butanol-saturated phenol. The aqueous phase was repeatedly extracted with phenol and RNA was precipitated with ethanol. Synchrony of embryos was examined at the cellular blastoderm and was normally about 70%. Unfertilized eggs were collected from a stock of the temperature-sensitive X chromosome-linked recessive lethal mutant F58, a gift of A. Garen. Each lane contains 30 μg, except lanes P1, which contain 23 μg, of RNA extracted from staged (25°C) animals. Lanes: (a) to 15-day-old pupae; A, 11- to 15-day-old adults. All stages are given in time after fertilization. Molecular weight markers in kb (a phage DNA digested with EcoRI and HindIII) are indicated on the left. Autoradiography was for 6 (a) or 66 (b) hr. Exposure times for the photographs of the autoradiograms were 15 (a) and 60 (b) sec. The bands evident in b in the interval containing transcripts approximately 2 kb long are presumably due to nonspecific binding of the probe to 18S and 28S ribosomal RNAs present in the samples.
\(N^{8114}, N^{8116}\) are derived from a known parental strain: Oregon R, which shows no detectable restriction site heterogeneity in the relevant region. Hence, we are confident that we have identified the structural basis for each of these \(Notch\) mutations even though we have not yet determined their respective genetic map positions.

Given the paucity of mutations for which parents and genetic map positions are known, we turned our attention to chromosomal rearrangements to compare physical and genetic data. However, the usefulness of such mutations in establishing a correlation between genetic and physical maps may be limited because localization of breakpoints in the genetic map can be impeded by the suppression of recombination in the vicinity of the rearrangement breakpoints (21, 22). In addition, it is possible that such breakpoints lie outside the locus yet cause a mutant phenotype through a position effect. However, Welschon and Keppy (5) found that recombination analysis near the breakpoints of chromosomal rearrangements involving only a few polytene bands is, in fact, possible. We have established the approximate physical locations of two such rearrangements, \(N^{76b8}\) and \(N^{53a1}\) (Fig. 3), which map in the vicinity of \(spl\) and \(fa\), respectively (5).

A correlation between physical and genetic map distances can be established from these data. Given that the distance between \(fa\) and \(spl\) is approximately 0.04 cM and that lesions associated with these mutations lie approximately 12 kb apart, it follows that 0.01 cM equals 3 kb (Fig. 3). This mapping relationship has been shown to be consistent for all testable intervals within the \(Notch\) locus. If we assume that recombination frequencies remain constant throughout the locus, we could argue that the genetic map distance occupied by the entire \(Notch\) locus (0.13 cM) corresponds to approximately 40 kb that map between \(-30\) and \(+12\) (Fig. 3). All mutations mapped in the present work lie within this region. We have identified the molecular lesions associated with 19 more \(Notch\) alleles, all of which map within the same 40-kb interval (data not shown).

Activity of the \(Notch\) locus is essential at various times throughout development. Several lines of evidence point to a 10.5-kb poly(A)\(^+\) RNA that is homologous to sequences altered in \(Notch\) mutants as an essential component for \(Notch\) function. Construction of germ line mosaics homozygous for a \(Notch\) mutation by Jimenez and Campos-Ortega (23) reveals the existence of a maternal component of \(Notch\) expression. Using probes homologous to the 10.5-kb poly(A)\(^+\) RNA, we detected transcripts in unfertilized eggs, as might be anticipated on the basis of these genetic data. The embryonic lethal period for conditional \(Notch\) alleles extends only through the first half of embryogenesis (23). The 10.5-kb RNA accumulates during the same period (4–12 hr) and falls off thereafter. It is interesting to note that the pattern of accumulation of this RNA also follows the pattern of mitotic activity observed for the neuroblasts in the developing embryo (24). Experiments involving conditional mutations indicate that \(Notch\) function is also required during larval and pupal stages (10). We find that 10.5-kb poly(A)\(^+\) transcripts are present during these developmental periods as well as during embryogenesis.

The molecular analysis we have described in this paper allows us to formulate a working hypothesis concerning the structure and expression of the \(Notch\) locus. We suggest that the entire \(Notch\) locus is represented by contiguous DNA sequences spanning an interval of approximately 40 kb. Furthermore, we propose that the mature 10.5-kb poly(A)\(^+\) RNA is a processing product derived from this region and is essential for the wild-type \(Notch\) function. This molecular model may provide an explanation for the genetic behavior of alleles within the locus.

The \(Notch\) locus is characterized by a complex pattern of complementation among a number of alleles that on the one hand exhibit diverse phenotypes and on the other behave as mutations within a single genetic unit (7). The possible existence of a single transcription unit that appears to be affected by all physically defined mutations within the locus would constitute a structural basis for these genetic observations. Such interpretation assumes that the 40-kb interval, which our data suggest constitutes \(Notch\), contains a single transcription unit. Moreover, it rests on the postulate that all sequences required for expression and function of the \(Notch\) product lie within the same interval. Yet we cannot, at present, exclude the possibility that transcripts arising from sequences flanking this interval play some role in \(Notch\) function. Detailed analysis regarding the physical structure and the transcriptional activity of the locus will be required to resolve the questions raised by our hypothesis.

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