Molecular analysis of the dominant homeotic *Antennapedia* phenotype

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Most of the dominant alleles of the homeotic gene *Antennapedia* (*Antp*) which show a transformation of antennae into legs are associated with large chromosomal inversions. To determine the molecular mechanisms underlying the dominant phenotype, one of the strongest *Antp* alleles (*Antp*^{73b}) was studied in more detail. The mutant chromosome has been cloned and the structure of the inversion has been identified. The inversion breaks two genes apart: the *Antp* gene and a previously unidentified gene, tentatively called *responsible for dominant phenotype* (*rfd*), located at 84D1-2. The two genes are transcribed in opposite directions and the breakpoints lie within introns of both genes. Through the inversion event, a reciprocal exchange of the first exons including promoters occurred leading to the production of new transcripts. The transcripts containing the entire *Antp* protein coding region which have been fused to the promoter of the *rfd* gene are lost in revertants of the dominant phenotype indicating a correlation between this fusion gene and the dominant phenotype. The molecular structure of inversion *Antp*^{73b} suggests that the dominant phenotype arises via ectopic expression of the normal *Antp* protein due to a gene fusion event.

Key words: *Antennapedia/Drosophila*/homeotic genes

Introduction

Homeotic genes in *Drosophila* are involved in the specification of segment identity. They were originally defined as genes in which a mutation can cause the transformation of one segment into another, or parts of a segment into parts of another segment. A large number of homeotic genes have been identified (Ouweneel, 1976; Gehring and Hiromi, 1986) and most of them are localized within two major clusters in the genome, the Bithorax-complex (BX-C; Lewis, 1978) and the Antennapedia-complex (ANT-C; Kaufman and Abbott, 1984). The BX-C genes have been shown to specify the development of segments posterior to the mesothoracic segment T2 (Lewis, 1978), whereas the ANT-C genes specify the more anterior segments (Kaufman and Abbott, 1984). The *Antennapedia* (*Antp*) gene is one member of the ANT-C and extensive genetic and developmental analyses have shown that *Antp* is required for the proper development of the mesothoracic as well as parts of the pro- and metathoracic segments (Wakimoto and Kaufman, 1981; Struhl, 1981; Schneuwly and Gehring, 1985; Sato *et al.*, 1985).

The recessive loss of function phenotype, which is due to a

deletion or inactivation of the *Antp* gene, shows a transformation of the meso- and metathoracic segment into more anterior, prothoracic structures (Wakimoto and Kaufman, 1981; Schneuwly and Gehring, 1985; Sato *et al.*, 1985). Localization of *Antp*⁺ transcripts by *in situ* hybridization (Levine *et al.*, 1983; Martinez-Arias, 1986) and of the *Antp*⁺ protein by immunofluorescence (J. Wirz and W.J. Gehring, in preparation) shows that the *Antp* gene is mainly active in those segments which are visibly affected by apparent null mutations. The *Antp* locus was initially identified by dominant mutations which show a transformation of the adult antennae into mesothoracic legs (LeCalvez, 1948). Most of the dominant alleles are homozygous embryonic lethal and show a recessive loss of function phenotype as described above. The dominant transformation is in the opposite direction of recessive loss of functional mutants and is thought to represent a dominant gain of function, since the phenotype can be 'reverted' by deleting the *Antp* gene (Denell, 1973; Struhl, 1981; Hazelrigg and Kaufman, 1983) and is dominant in triploids (Tottoli, 1978).

The structural organization and the sequence of the *Antp* genes has been analyzed recently (Schneuwly *et al.*, 1986). The gene is composed of two promoters, eight exons spanning more than 100 kb, and two termination-processing regions. Four major polyadenylated transcripts were found, two of them starting at an internal promoter in front of exon 3. Most of the dominant alleles contain large chromosomal inversions, in which one breakpoint lies between exons 1 and 2 or exons 2 and 3, disrupting the normal gene structure (Garber *et al.*, 1983; Scott *et al.*, 1983; Schneuwly *et al.*, 1986).

Understanding the molecular mechanism which generates the dominant gain of function phenotype might provide some clues about the function of the *Antp* gene. Therefore, we decided to clone and characterize the mutated *Antp* gene of one of the strong *Antp* alleles (*Antp*^{73b}). We report the cloning and the structure of the inversion. The inversion is flanked by two repetitive elements in opposite orientations. Transcriptional analysis around both breakpoints in *Antp*^{73b} shows that the inversion leads to a gene fusion in which the entire *Antp* protein coding region has been placed under a new promoter. The absence of these fusion transcripts in *Antp*^{73b} 'revertants' suggests that they are responsible for the dominant phenotype.

Results

Isolation and molecular characterization of the chromosomal inversion breakpoints of Antp^{73b}

The *Antp*^{73b} mutation is associated with an inversion which spans the chromosomal region 84B1-2 to 84D1-2 (Garber *et al.*, 1983). The inversion breakpoints were more precisely localized by hybridizing nick-translated genomic subclones to Southern blots of genomic DNA digested with various restriction enzymes. Figure 1 shows a Southern blot using a fragment which spans the *Antp*^{73b} inversion breakpoint. In heterozygous *Antp*^{73b} DNA, new fragments are present which indicate a genomic rearrangement. DNA isolated from *Antp*^{73b}/TM3 flies was used to con-

Fig. 1. Whole genome Southern blots using *Antp*^{73b} DNA. Total genomic DNA from wild-type (Oregon-R, lanes 3 and 6) or *Antp*^{73b}/TM3 (lanes 2 and 5) and from *Antp*^{73b+R1}/TM3 (lanes 1 and 4) was either digested with *SalI* (lanes 1–3) or *EcoRI* (lanes 4–6). Nick-translated DNA from plasmid 372RB spanning the *Antp*^{73b} breakpoint was used as a probe. The arrows indicate bands, which appear only in *Antp*^{73b} and *Antp*^{73b+R1} DNA due to the inversion breakpoints. The weak band in Oregon-R (lane 6) is probably due to restriction site polymorphism.

struct a genomic library and phages were isolated which hybridized to the breakpoint specific fragments. Three classes of phages were isolated: one class containing inserts spanning the 84B1-2 inversion breakpoint, another class spanning the 84D1-2 breakpoint and a third class containing inserts which were derived from the balancer chromosome. In Figure 2a and b the restriction maps of the various isolated phages and their homology to the *Antp* region is shown. The inversion separates the first exon from the rest of the gene as shown in Figure 2d. The inversion is flanked by repetitive elements which are arranged in opposite orientations to each other (see below). Non-repetitive DNA, which hybridizes to the 84D1-2 region was used to clone corresponding phages from a wild-type library (Canton-S; Maniatis *et al.*, 1978) as shown in Figure 2c. This enabled us to carry out detailed structural and transcriptional analysis around the two different breakpoints.

Molecular structure and partial sequence of Doc, a repetitive element associated with the Antp^{73b} inversion

From the restriction map of the two inversion breakpoints (see Figure 2) it was obvious that an ~4 kb repetitive long DNA fragment was present at both breakpoints. Using a subcloned fragment from this region, multiple bands were detected by hybridiz-

Fig. 2. (a) Restriction map of the 84B1-2 breakpoint. Phages An15 and An26 were isolated from the *Antp*^{73b}/TM3 library (see Materials and methods) and span the 84B breakpoint. The region homologous to the *Antp* walk is shown by a broken line. The first *XhoI* (X) site corresponds to position 81 in the *Antp* walk (Garber *et al.*, 1983). The repetitive Doc element is indicated. The arrow marks the breakpoint. (b) Restriction map of the 84D1-2 breakpoint. Phages An7 and An10 were isolated in the same screen as the phages shown in (a). They span the 84D breakpoint. The broken line shows the homology to the *Antp* walk where the *SalI* site maps at position 63 (Garber *et al.*, 1983). The arrow marks the breakpoint. (c) Restriction map of the 85D wild-type DNA. CS20 and CS101 are two overlapping phages from the Canton-S library (see Materials and methods). The arrow marks the breakpoint. (d) Structure of the *Antp*^{73b} inversion. The inversion is flanked by two Doc elements in opposite orientation. The first exon of *Antp* has been separated from the rest of the gene and the transcription orientation is now in the opposite direction (not drawn to scale). Abbreviations: B, *Bam*HI; H, *Hind*III; R, *Eco*RI; S, *Sal*I; X, *Xho*I.

ation to whole genome Southern blots or to giant chromosomes (data not shown). As estimated from the number of bands on giant chromosomes, this sequence seems to be present in ~40 copies per genome (not counting those copies present in heterochromatin) which is typical for middle repetitive sequences like the *copia* family (Spradling and Rubin, 1981).

Different known repetitive sequences were hybridized to Southern blots containing the breakpoint phages (data not shown), which allowed us to identify the repetitive sequence as a Doc element, originally isolated by Bender *et al.* (1983). To determine the structure of these elements, all four ends of the two Doc repeats were sequenced. The structure and sequence is shown in Figure 3. The Doc elements do not have long terminal repeats. Instead, there is a long poly(A) stretch at one end of the elements. The sequences of the two elements differ by only 21 bp at the 5' end. These findings show that the Doc element is more related to F elements than to the *copia* family (DiNocera *et al.*, 1983). Many of the middle repetitive elements including F elements generate target site duplications of a few bases. As shown in Figure 3, the two Doc elements at 84B1-2 and 84D1-2 generate

Fig. 3. Structure and partial sequence of the Doc elements. (a) Orientation of the two Doc elements and different sequenced regions. (b) Sequence of the Doc elements. In lines (1) and (2) the 5' ends of the two Doc elements are aligned whereas in lines (3) and (4) the 3' ends are aligned. Note that the Doc element at 84D1-2 is 21 bases longer and that both elements contain a poly(A) stretch at the 3' end. The insertion region at 84B1-2 and 84D1-2 has been sequenced from wild-type DNA (Canton-S) and is aligned with the flanking regions of the elements. Note the target site duplication of 6–7 bases at 84B and 12–14 bases at 84D. Ambiguities are due to the poly(A) stretch at the 3' end. 400 bases at both ends of the elements have been sequenced and are perfectly homologous to each other (data not shown).

target site duplications. However, these target site duplications are separated such that each Doc element is flanked by its original target site on one end and a target site from the second element on the other end. The structure of these two Doc elements and the location of the target site duplications strongly suggest that the inversion has been generated by an homologous recombination event between the two Doc elements.

Transcriptional analysis of the *Antp* gene in *Antp*^{73b} mutants

Knowing that the inversion in *Antp*^{73b} changes the gene structure of *Antp* one would also expect to see a change in the transcriptional pattern of this gene. Hybridizing exon-specific DNA probes to Northern blots of mutant RNA, it should be possible to analyze different transcripts derived from both sites of the inversion. RNA was fractionated on a 0.8% agarose gel, transferred to nitrocellulose filters and hybridized with a nick-translated DNA probe derived from exon 1. The results are shown in Figure 4A. In RNA derived from pupae heterozygous for the *Antp*^{73b} mutation an additional 4.3-kb transcript appeared (see lane 3). This shows that exon 1, which is 1 kb long, is fused to sequences in the 84D1-2 region forming a stable polyadenylated mRNA. The developmental profile of the mutant 4.3-kb transcript is exactly the same as for *Antp*, which would be expected if the promoters were identical (data not shown). An identically treated nitrocellulose filter was hybridized using a fragment containing exons 4–8. These results are shown in Figure 4B. In RNA derived from *Antp*^{73b} pupae (lane 3) two new bands of 4.6 and 6.2 kb appear. Since the *Antp* promoter of the first exon has been separated by the inversion event, there must be a new promoter element responsible for the transcripts seen here. The developmental profile of these two mutant transcripts is different from *Antp* in that it is not expressed during embryogenesis (data not shown). No additional transcripts have been identified using exon 3 as a probe indicating that the second promoter is not responsible for mutant transcripts (data not shown). To determine the difference between the 4.6- and 6.2-kb transcripts, the

Fig. 4. Northern blot analysis of *Antp* mutant transcripts. Each lane contains 40 μ g poly(A)⁺ RNA from pupae. Lane 1, *Antp*^{73b+R2}/TM1 RNA; lane 2, *Antp*^{73b+R1}/TM3 RNA; lane 3, *Antp*^{73b}/TM3 RNA; lane 4, wild-type (Oregon-R) RNA. (A) Nick-translated probe derived from exon 1. (B) Nick-translated probe derived from exon 1. The mutant transcripts are labelled with arrows whereas the wild-type transcripts are labelled with lines.

same Northern blots were hybridized with a probe which is specific for the large *Antp* trailer sequences. This probe recognizes only the 5.0-kb *Antp* transcript in RNA from wild-type pupae (Schneuwly *et al.*, 1986). In RNA from heterozygous *Antp*^{73b} pupae, it only recognizes the 5.0- and the 6.2-kb transcripts. This indicates that the difference in size between the 4.6- and 6.2-kb mutant transcripts is due to the same alternative use of different polyadenylation signals as in *Antp*⁺ transcripts (Schneuwly *et al.*, 1986).

Two independent revertants of the *Antp*^{73b} mutation (*Antp*^{73b+R1} and *Antp*^{73b+R2}) have been found spontaneously in an *Antp*^{73b} fly stock (M.M.Green, personal communication). These revertants have lost the dominant antennae to leg transformation but they still share the recessive *Antp* lethality. Heterozygous RNA from these revertants was analyzed on the same gels as the *Antp*^{73b} RNA. As shown in Figure 4 (lanes 1 and 2), both revertants still show an additional 4.3-kb transcript using an exon 1-specific probe. Interestingly, using a probe which contains exons 4–8, the mutant transcripts of 4.6 and 6.2 kb are no longer present. In the case of one revertant (*Antp*^{73b+R2}), the loss of fusion transcripts is due to a new chromosomal inversion breakpoint, which destroys the 84B1-2 gene fusion (data not shown). This shows that the reversion event is associated with the loss of the additional transcripts and suggests that they are responsible for the dominant phenotype. The fact that the protein coding portion of *Antp* is not affected by the inversion and is contained within these two mutant transcripts makes it very likely that they still produce a functional *Antp* gene product. However, since these transcripts are under a new control, the tissue specificity of these

Fig. 5. Restriction map and structure of the *rfd* gene. (a) Restriction maps of different cDNAs containing parts of the *rfd* transcription unit. (b) Summarized map based upon Southern hybridizations and heteroduplex mapping. The inversion breakpoint lies in the first intron as indicated by the arrow. Transcription orientation is from left to right (see Figure 2).

transcripts might be altered and might lead to the expression of the *Antp* protein in the wrong places, e.g. in the antennal anlagen.

Molecular characterization of the 84D1-2 inversion breakpoint region of *Antp*^{73b}

What are the molecular mechanisms generating the mutant transcripts? It is possible that the mutant fusion transcripts are initiated from the promoter of a different gene, which is located at the inversion breakpoint at 84D1-2. In order to analyze the structure of any RNA transcripts that may have arisen from this region, wild-type pupal cDNA libraries were screened using the entire breakpoint region at 84D1-2 (Figure 2c) as a probe. The results are shown in Figure 5. Two different cDNAs were isolated that contained inserts of 3.8 and 5 kb, indicating that there is a transcription unit located in this region. The structure of this gene, tentatively called *rfd* (responsible for dominant phenotype), was further analyzed by Southern hybridizations and heteroduplex-analysis with genomic phages. The results are shown in Figure 5B. The gene is composed of several exons and introns and the *Antp*^{73b} inversion breakpoint (see arrow) is located in an intron which separates exon A from all the other exons.

Transcription orientation was determined by Northern blot analysis using cDNA4 as a probe, as described in the legend to Figure 6. Single-stranded ³²P-labeled RNA was hybridized to pupal RNA of wild-type and *Antp*^{73b}/TM3 origin. In wild-type RNA, two transcripts of 6.2 and 5 kb were detected. The transcription orientation places the 5' end in exon A. Since the inversion breakpoint in *rfd* is located between exon A and B, and since this gene is transcribed in opposite orientation to *Antp*, the inversion event leads to a reciprocal fusion of introns from both genes. At the 84B1-2 breakpoint exon A is placed upstream of exons 2–8 of *Antp* whereas at the 84D1-2 breakpoint, exon 1 of *Antp* is found upstream of exons B–E. Thus, the two new *Antp* transcripts seen in mutant RNA (see Figure 2) are probably products of these two fusion genes. Indeed, as shown in Figure 6, Northern blots of *Antp*^{73b} RNA reveal an additional weak band with a size of 4.6 kb. This band corresponds in size to one of the fusion transcripts detected with the *Antp* exon 4–8 probe. The second fusion transcript, which is 6.2 kb long, cannot be separated from the original transcript of this gene because of the similar size.

Discussion

The major findings of this work are summarized in Figure 7. Molecular and transcriptional analyses of both inversion break-

Fig. 6. Northern blot of gene *rfd* in wild-type (lane 1) and *Antp*^{73b}/TM3 RNA (lane 2). Each lane contains 40 µg of poly(A)⁺ RNA of pupal stages. The insert from cDNA4 was placed under control of a bacteriophage SP65 RNA polymerase promoter. RNA was synthesized using SP6 polymerase in the presence of [α -³²P]ATP and hybridized to the Northern blot. The weak but reproducible extra band in mutant RNA is indicated by an arrowhead.

Fig. 7. Structure of the *Antp*^{73b} inversion. The schematic map is not drawn to scale. Exons derived from *Antp* are numbered 1–8 whereas exons derived from *rfd* are labelled A–E.

points in the dominant *Antp* allele *Antp*^{73b} show that the mutations lead to additional transcripts containing the whole *Antp* open reading frame. These new transcripts were generated by an inversion event. The inversion itself is flanked at both ends by repetitive elements (Doc) which are present in opposite orientation to each other. Sequence analysis of the ends of these elements shows that they are structurally related to F elements, which contain poly(A) stretches and no long terminal repeats (DiNocera *et al.*, 1983). The inversion disrupts the *Antp* gene within the first intron and separates the putative upstream controlling region and the first exon from the protein coding portion of the gene. At the second breakpoint, a previously unidentified gene (*rfd*), is also disrupted in the first intron. The transcription orientation of this gene is opposite to that of *Antp* and therefore allows for a reciprocal exchange of the promoters and first exons of each of these genes. This reciprocal exchange leads to new transcripts that are present in heterozygous *Antp*^{73b} RNA. Transcripts derived from the *Antp* promoter containing exon 1 of *Antp* and exons B–E of *rfd* are still present in *Antp* 'revertants' and are therefore not related to the dominant phenotype. Mutant transcripts coding for the *Antp* gene product, which are fused to exon A and transcribed under the control of the *rfd* gene are lost in both *Antp* 'revertants' and therefore are related to the dominant phenotype. The molecular basis of the dominant pheno-

Fig. 8. Model for the generation of a chromosomal inversion. (1) The putative parental chromosome contains two homologous repetitive elements inverted orientation. (2) Intra-chromosomal pairing between the two homologous elements. (3) Recombination leads to an inversion of the chromosomal segment between the two elements.

type seems to be the inappropriate expression of the *Antp* protein coding region under a new promoter.

How can such an inversion be generated? The fact that the inversion is flanked by two repetitive elements in opposite orientations suggests the following model (Figure 8). The parental chromosome, which unfortunately is not available for *Antp*^{73b}, contains both elements in inverted orientation. Since both elements are located in introns, they presumably do not affect gene activity. Pairing of the homologous sequences and homologous recombination between the two repetitive elements leads to an inversion of the whole chromosome segment between the two elements. This model is strongly supported by the fact that the target site duplications of the two *Doc* elements at 84B1-2 and 84D1-2 are separated as shown in Figure 4 indicating that they have been exchanged. This can only be explained by a recombination event within the *Doc* elements. If the two elements had been in the same orientation, homologous recombination would have led to a deletion and the corresponding duplication (Goldberg *et al.*, 1983). This is of evolutionary interest because it shows that gene duplications and exon shuffling can occur where especially exon shuffling might lead to new gene functions as is shown by the dramatic change of an antenna into a leg.

As discussed above, the inversion in *Antp*^{73b} leads to an exchange of promoters which seems to be the cause of the dominant phenotype. All of the known dominant *Antp* alleles, which are homozygous lethal, correspond to chromosomal inversions or translocations and it is proposed that the model (described above) can be generalized for all of these *Antp* alleles. This is strengthened by the fact that none of the inversions disrupt the protein coding portion of *Antp*. There are two exceptional alleles, *Nasobemia* and *Antp*^{72j} (Gehring, 1966; Baker, 1972), which are homozygous viable. In *Nasobemia*, there is an insertion element which has integrated into the third intron (data not shown; Scott *et al.*, 1983; Schneuwly *et al.*, 1986). This element, which

belongs to the *copia*-like elements (Spradling and Rubin, 1981) could provide a new promoter, for example its long terminal repeat, which would lead to inappropriate expression of the *Antp* gene product.

An important question which has to be answered concerns the specificity of the foreign promoters in different *Antp* alleles. One might assume that these promoters are only active in the eye antennal anlagen since the phenotype is so specifically restricted to the head. However, all *Antp* alleles which have been analyzed so far have a different second inversion breakpoint and therefore it seems unlikely that the inversion always hits an antenna-specific gene. It seems more plausible that these promoters are relatively unspecific but that only the eye antennal anlagen are sensitive to ectopic expression of the *Antp* gene. Instead of rigorously analyzing the tissue specificity of the foreign fused promoters from different *Antp* alleles, we decided to test this hypothesis more directly by *in vitro* construction of a mutant gene containing a heat-inducible promoter fused to the *Antp* cDNA coding for the entire *Antp* protein which is, after heatshock, expressed in all tissues. Indeed, if this construct is reintroduced into the *Drosophila* germ line by P element transformation, such transformed flies show, after a heatshock at the early third larval stage, a transformation of antennae into legs (Schneuwly *et al.*, in preparation). Although the *Antp*⁺ protein is expressed in all cells in these transformed flies, only the head is sensitive to ectopic expression of the *Antp*⁺ protein. This supports our idea that it is not the specificity of the foreign promoter but rather the sensitivity of the target tissue which leads to such a specific homeotic phenotype as the antenna to leg transformation.

Materials and methods

General DNA techniques

Restriction endonuclease digestions, nick-translations, screening of bacteriophage λ libraries, ligations and transformations were performed following the protocols described in Maniatis *et al.* (1982). Plasmid DNA preparations were done according to Frei *et al.* (1985), phage DNA and genomic DNA from *Drosophila* were isolated according to Garber *et al.* (1983) and DNA fragments were isolated using a freeze-squeeze method described by Thuring *et al.* (1975). Heteroduplex analysis of DNA-DNA hybrids in the electron microscope was performed as described by Brack (1981).

Southern blot analysis

Fragments separated on agarose gels were transferred to nitrocellulose filters according to the procedure of Smith and Summers (1980). Filters were pre-hybridized in 50% formamide, 5 $\times$ SSPE, 5 $\times$ Denhardt's, 0.2% SDS, 0.1% Na-pyrophosphate and 200 μ g/ml denatured salmon sperm DNA for 4–6 h at 42°C. Hybridization with nick translated probes (10^6 – 10^7 c.p.m./ml) was done overnight at 42°C in the same buffer. Filters were washed four times for 10 min in 2 $\times$ SSC, 0.2% SDS at room temperature and twice for 30 min at 68°C.

Construction of genomic libraries

Genomic DNA from *Antp*^{73b}/TM3 flies was isolated as described by Garber *et al.* (1983). Twenty-five micrograms of DNA was partially digested with *Sau3A*I and separated on a 0.4% agarose gel. Fragments in the size range 15–23 kb were cut out and eluted as described by Thuring *et al.* (1975). These fragments were mixed with *Bam*HI cut λ -vector DNA (EMBL 4; Frischauf *et al.*, 1983), ligated and packaged *in vitro* using standard protocols (Hohn, 1979). Phages containing inserts were selected by the *Spi*⁻ phenotype (Frischauf *et al.*, 1983). The yield was 10^5 independent phages. As a source of wild-type DNA, the Canton-S library of Maniatis *et al.* (1978) was used.

RNA isolation and Northern blot analysis

Embryonic RNA was extracted by the hot phenol method (Lepesant *et al.*, 1978). Larval and pupal RNA was extracted by the guanidium isothiocyanate-hot phenol method (Maniatis *et al.*, 1982) after pulverizing frozen larvae or pupae with a pestle and mortar in liquid nitrogen. Poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography (Collaborative Research Inc. Type 3). Forty micrograms of poly(A)⁺ RNA from various stages were fractionated on 1.0% agarose formaldehyde gels (Maniatis *et al.*, 1982). The RNA was directly transferred to nitrocellulose filters overnight in 20 $\times$ SSC by the method of Southern (1977).

Hybridization was performed in 50% formamide, 10% dextran sulfate, 50 mM Tris-HCl (pH 7.5), 10 × Denhardt's solution (Denhardt, 1966), 1.0 M NaCl, 0.5% sodium pyrophosphate and 250 µg/ml yeast RNA at 42°C overnight. The nick-translated probe was added at a concentration of 5×10^6 c.p.m./ml. Filters were washed successively four times with $2 \times$ SSC, 0.3% SDS at room temperature for 10 min and then twice at 68°C for 30 min. Single-stranded probes were prepared using SP6 RNA polymerase as recommended by the manufacturer (Promega, Biotec).

DNA sequencing

DNA sequencing was performed using the standard dideoxy chain termination procedure (Sanger *et al.*, 1977). Restriction fragments were subcloned in appropriate M13 vectors (Messing, 1983).

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