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A genetic approach to the dissection of P transposable element functions in *Drosophila*

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We have made a P element derivative that carries the selectable marker gene *rosy*, but which acts like a non-defective, intact P element. When introduced into an M strain fly, this element continues to transpose autonomously within the genome, and elicits certain other characteristics of hybrid dysgenesis. By mutagenizing the P element *in vitro*, and assaying its activity *in vivo*, both singly and in combination with other P elements, we have tentatively identified the regions of the element encoding the transposase function. In addition, we have examined the pattern of poly(A)$^+$ transcripts encoded by P elements of natural P strains, and by our P element marked with *rosy* in transformed flies, and find two species that appear to be derived from non-defective elements, one of which may be the message for transposase.

Introduction

The mechanism and regulation of transposition of many prokaryotic transposable elements, though complex and multifarious, is well understood, largely because these elements carry selectable markers (e.g. antibiotic resistance) and because they can be manipulated both *in vitro* and *in vivo*. In contrast, the molecular biology of metazoan transposable elements is limited almost exclusively to structural description of the different elements and their transcripts. Progress in this field has been hampered by inherent difficulties in studying a multigene family, and by lack of useful assays for detecting transposition of an element within the genome.

P elements, the family of mobile genetic elements that are responsible for the phenomenon of P–M hybrid dysgenesis in *Drosophila melanogaster*, are a particularly interesting class of transposable elements, because their mobility has been shown to be under genetic control. (For review on P elements see Engels (1983); for review on hybrid dysgenesis see Bregliano & Kidwell (1983)). When the elements are quiescent they are said to be in the P cytotype (Engels 1979*b*), the cellular environment of P strain flies. P cytotype is apparently determined by the P factors themselves (Engels 1979*a*). When P elements are carried by a sperm into an egg of the M cytotype (the permissive environment characteristic of M strain flies, which usually lack functional P elements), hybrid dysgenesis ensues in the germ line of the developing embryo. The dysgenic hybrid may show a series of genetic aberrations, including chromosomal rearrangements, visible and lethal mutations, male recombination, and a high level of gonadal sterility. In the reciprocal cross, between an M strain male and a P strain female, or a P × P cross, the P elements are maintained in the P cytotype, and no hybrid dysgenesis is observed.

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A number of P elements have been cloned (Rubin *et al.* 1982) and sequenced (O'Hare & Rubin 1983). Some share a conserved 2.9 kilobase structure corresponding to the intact non-defective P factors, and are believed to encode genes for transposition and cytotype determination. Others are smaller, defective elements, missing sequences internal to the terminal inverted repeats that flank the element. The functional integrity of the 2.9 kilobase element has been directly demonstrated by the experiments of Spradling & Rubin (1982), who showed that a cloned, intact element supplies a *trans*-acting function required for both its own transposition and for the transposition of defective non-autonomous P elements. These observations led to the P element-mediated gene transfer system in *Drosophila* (Rubin & Spradling 1982).

A genetic approach to dissecting the functions encoded by P elements requires that one should be able to examine a single element of defined structure, rather than the heterogeneous population of defective and non-defective P elements found in the genomes of natural P strains. Additionally, the element needs to be marked genetically, so that its presence and location in the genome of a living fly can be quickly detected, independently of the element's ability to produce signs of hybrid dysgenesis.

We have made a P element derivative that carries the genetic marker gene *rosy*, but otherwise behaves as a non-defective 2.9 kilobase element. We have used this marked P element to begin a genetic dissection of P element functions.

Results

Construction of an autonomous P *element marked with rosy*

We positioned a DNA fragment of 7.2 kilobases containing the wild type *rosy* gene in a non-coding region of the 2.9 kilobase P element, in a manner that we hoped would be least likely to perturb element-encoded functions required in *cis* or *trans*. The resultant construct (figure 1) contains 2884 of the 2907 bases of the complete P element unrearranged. It lacks 23 base pairs (b.p.) of the 31 b.p. right hand inverted repeat, and has instead a sequence of 7.2 kilobases containing the *rosy* gene. The final 223 bases of P are duplicated at the end of the fragment containing the *rosy* insert, to supply an intact right hand repeat.

The marked P element, called PcR[*ry*], and a derivative, Pc[*ry*] (modified by the removal of certain non-essential restriction nuclease cleavage sites flanking the *rosy* gene), were tested

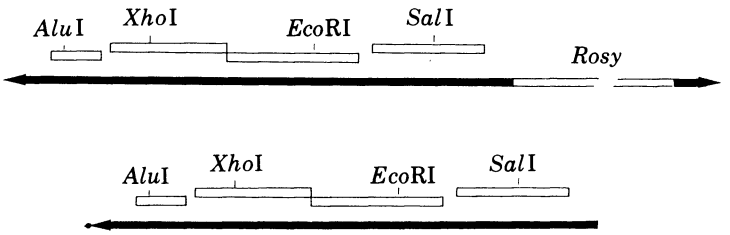

FIGURE 1. Schematic map of Pc[*ry*] and its derivatives (above), and the transposition-defective P element in pπ25.7wc (below). The black bar represents P element sequences; arrows represent the 31 basepair inverted repeats. Note that the right hand inverted repeat of pπ25.7wc ('wings clipped') has been removed. The boxes above the elements indicate the extents of the four major open reading frames. The restriction nuclease sites indicated within each reading frame of Pc[*ry*] are the points at which frameshift mutations have been introduced into the sequence, to generate the derivative elements Pc[*ry*]Alu, Pc[*ry*]Xho, Pc[*ry*]RI, and Pc[*ry*]Sal. The open box region of the Pc[*ry*] element represents the sequences containing the *rosy* gene.

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for their ability to transpose autonomously (that is, without the aid of a helper element) from the plasmid to the germ line chromosomes when injected into M strain ry$^-$ embryos. They were found to transpose at a rate comparable with that seen for other P element constructions transposed with the aid of helper elements. By this criterion, Pc[*ry*] and PcR[*ry*] behave as autonomous, non-defective P elements. A line of transformed flies was established from each of the five independent events (table 1): Pc[*ry*].2, Pc[*ry*].17c, Pc[*ry*].11, Pc[*ry*].7, and PcR[*ry*].81.

TABLE 1. TRANSFORMED LINES CONTAINING MARKED P ELEMENTS

name†	site of lesion	chromosome location
PcR[*ry*].81	none	X
Pc[*ry*].2	none	II
Pc[*ry*].7	none	III
Pc[*ry*].11	none	III
Pc[*ry*].17c	none	III
Pc[*ry*]Alu.18	ORF 0	III
Pc[*ry*]Xho.11	ORF 1	II
Pc[*ry*]Xho.26	ORF 1	II
Pc[*ry*]Xho.41	ORF 1	X
Pc[*ry*]RI.8	ORF 2	X
Pc[*ry*]RI.28	ORF 2	III
Pc[*ry*]Sal.1	ORF 3	III

† The number following the decimal point refers to the particular transformed line. For example, Pc[*ry*]RI.8 and Pc[*ry*]RI.28 are two independently derived transformant lines bearing the same P element construction.

Establishment of Drosophila lines containing single mutagenized Pc[*ry*] *elements*

Having demonstrated that Pc[*ry*] can autonomously transform *Drosophila*, we next asked what mutations would destroy its ability to transpose. The four open reading frames (ORFs), found by DNA sequencing (O'Hare & Rubin 1983) were obvious candidates for P element-encoded genes. We created frameshift mutations in each of the ORFs by altering appropriate restriction endonuclease cleavage sites. The four mutagenized Pc[*ry*] elements are designated Pc[*ry*]Alu, Pc[*ry*]Xho, Pc[*ry*]R1, and Pc[*ry*]Sal, and carry frameshift mutations in ORFs 0, 1, 2, and 3, respectively (figure 1).

When attempting to introduce a mutagenized Pc[*ry*] element into the *Drosophila* genome, it is necessary to supply a functional transposase activity in *trans* from a helper P element, since a lesion created in the P sequence of Pc[*ry*] might disrupt this activity. A potential problem arises if the mutagenized Pc[*ry*] element and the wild type (wt) helper element both integrate into the fly genome, for the presence of the wt element would mask the effect of the mutation. Separating the two elements genetically, while possible, would be extremely tedious.

To circumvent this problem, a modified helper P element was constructed which we call pπ25.7wc (for 'wings clipped'). It is derived from the autonomous P element in the plasmid pπ25.7, by removal of the last 23 b.p. of the 31 b.p. right hand inverted repeat, leaving the remainder of the element intact.

By using pπ25.7wc to supply the helper function, we could transform *Drosophila* with each of the different Pc[*ry*] derivatives without regard to the integrity of its transposase gene. We established one or more *Drosophila* lines, each containing a single element, as judged by DNA blot hybridization of the genomic DNA from the transformed flies, for each of the four different Pc[*ry*] mutant constructions.

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The Pc[*ry*] *element continues to transpose to new sites within the genome of the transformed fly*

A characteristic behaviour of P elements is their high level of excision and transposition to new chromosomal sites during dysgenesis (Bingham *et al.* 1982). One line, PcR[*ry*].81, transformed with an unmutagenized element, and two lines bearing different mutagenized derivatives, Pc[*ry*]Xho.41 and Pc[*ry*]Rl.8 (representing mutations in ORF 1 and ORF 2 respectively), carried the marked P element on the X chromosome. The rates of excision and transposition of the P elements of these three lines were examined by searching for males that had lost the ry^+ phenotype and females that had acquired the ry^+ phenotype, from among the offspring of flies maintained in attached-X, *ry* stocks. In such a stock, the two X chromosomes of the female segregate as a unit. The only viable offspring are males receiving the free X from their fathers, and females carrying the attached-X from their mothers. Since the PcR[*ry*] element resides on the free X, all the males should be ry^+ and all the females ry^-. Excision of the element from the X in the germ line of the F1 male will produce a ry^- male gamete. Similarly, the transposition of the element to an autosome could produce a ry^+ female gamete.

Using this test, we found that loss of ry^+ occurred in approximately 1.6% of the X chromosomes; transpositions of ry^+ to the autosomes occurred at a rate of about 2.4%. Sublines were established from several separate ry^+ attached-X females, and were examined by DNA blotting. Each line showed a second band in addition to the one present in the parental PcR[*ry*].81 line, confirming that a transpositional event has resulted in a second integration site on an autosome (data not shown).

Loss of the ry^+ phenotype in males does not itself mean the PcR[*ry*]element has excised. From three of the ry^- males we established sublines (again maintained with attached-X females), and examined the DNA of these lines by blot hybridization. One ry^- male had lost the band characteristic of the PcR[*ry*].81 integration site, and was devoid of sequences hybridizing to the P element probe. However two other ry^- males have apparently retained at least some of the P element sequences.

In contrast with the results with the wt PcR[*ry*] element, we found no evidence for excision or rearrangement of the mutant P element derivatives in line Pc[*ry*]Xho.41 or in line Pc[*ry*]Rl.8, among over 2000 male offspring scored, and transpositions among about 1000 female progeny scored. This indicates that the ORF 1 and ORF 2 lesions in these two P element derivatives are each sufficient to eliminate (or at least greatly reduce) the excision, rearrangement, and transposition seen with the wild type P element.

The wild type Pc[*ry*] *can destabilize singed-weak, but its mutant derivatives cannot*

Singed-weak (sn^w) is a hypermutable allele of the *singed* bristle locus on the X chromosome. It arose in the offspring of a dysgenic hybrid (Engels 1979*b*) and its phenotype results from the presence of two small, defective P elements at the *sn* locus (H. Roiha, K. O'Hare and G. Rubin, unpublished). In the offspring of a P♂ by M♀ dysgenic cross involving sn^w, up to 50% of the gametes of the F1 dysgenic hybrids no longer carry of the parental sn^w allele. One or the other of the two defective P elements at sn^w excises, generating one of two new phenotypes in the F2 offspring: a much more extreme singed bristle (sn^e) and an apparently wild type bristle (sn^+). However, when maintained in the P cytotype, or when maintained in a genome devoid of all other P elements (and therefore M cytotype), the sn^w allele is essentially stable. Thus the

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destabilization of sn^w is an extremely sensitive assay for the presence of an activity (presumably transposase) provided by functional P elements. A single autonomous P element is sufficient to induce sn^w instability (Spradling & Rubin 1982).

We tested the ability of Pc[*ry*] and its mutant derivatives to destabilize sn^w. The putative P male carrying the Pc[*ry*] element is crossed to an M strain female homozygous for sn^w (and *ry*). If the transformed line is heterozygous for the chromosome bearing the Pc[*ry*] element, then half the F1 offspring will be ry^+ and be potential dysgenic hybrids and half will receive the other chromosome and be ry^-. These ry^- individuals serve as controls of nearly identical genetic composition. The ability of the Pc[*ry*] to destabilize the sn^w allele in the germ line of these F1 hybrids was assayed by crossing the F1 sn^w males to tester females homozygous for sn^3, a recessive allele of the *singed* locus. The phenotypes of the F2 female offspring will be exclusively sn^w if no sn^w destabilization has occurred, but if the Pc[*ry*] element is capable of providing transposase activity some will be sn^+ or sn^e.

All four wild type Pc[*ry*] lines tested were able to induce the destabilization of sn^w to sn^e (table 2), (while sn^+ derivatives were observed in these experiments, they were not scored separately because of the difficulty in reliably distinguishing the sn^w and sn^+ phenotypes). None of the tested ry^- siblings did so, confirming that the presence of the Pc[*ry*] element was required for sn^w destabilization to occur.

Table 2. Destabilization of *singed-weak* by Pc[*ry*] and its derivatives

	F1 progeny†		F2 progeny		
strain	total number tested	number showing germline sn^w instability	total	sn^e	
Pc[*ry*]Alu.1	25	0	1627	0	
Pc[*ry*]Xho.11	42	0	2357	0	
Pc[*ry*]Xho.26	13	0	761	0	
Pc[*ry*]Xho.41	18	0	1864	0	
Pc[*ry*]Rl.8	19	0	2505	0	
Pc[*ry*]Rl.28	19	0	1514	0	
Pc[*ry*]Sal.1	40	0	2509	0	
PcR[*ry*].81	17	6	1290	22	(3.4+1.9%)‡
Pc[*ry*]2	20	14	1618	131	(8.1+3.6%)
Pc[*ry*]11	20	13	1672	28	(1.7+0.4%)
Pc[*ry*]17c	24	8	1242	32	(2.6+1.0%)
Pc[*ry*]Xho.41 + Pc[*ry*]Rl.28	25	0	5249	0	
Pc[*ry*]Xho.41 + Pc[*ry*]Sal.1	25	0	5136	0	
Pc[*ry*]Xho.41 + Pc[*ry*]Alu.18	24	0	5470	0	
Pc[*ry*]Rl.8 + Pc[*ry*]Sal.1	25	0	5319	0	
Pc[*ry*]Rl.8 + Pc[*ry*]Alu.18	25	0	6075	0	
Pc[*ry*]Sal.1 + Pc[*ry*]Alu.18	17	0	3352	0	
π2§	25	25	2388	277	(11.6+2.4%)
ry^- siblings‖	16	0	975	0	

† Individual males either homozygous or heterozygous for the chromosome bearing the marked P elements were crossed to sn^w; *ry* (M) females. The F1 offspring were examined for evidence of sn^w destabilization in their germ lines by crossing them to flies homozygous for sn^3.

‡ Standard errors were determined by the method of Engels (1979*c*).

§ π2 is a strong, naturally occurring P strain, serving as a positive control.

‖ The offspring that did not inherit the chromosome bearing the Pc[*ry*] element from crosses involving transformed lines heterozygous for the element, serving as negative controls.

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In contrast with the wild type Pc[*ry*] elements, none of the mutant derivatives of Pc[*ry*] showed any evidence of inducing sn^w instability (table 2). This indicates that each of the four different ORF lesions in the elements Pc[*ry*]Alu, Pc[*ry*]Xho, Pc[*ry*]Rl, and Pc[*ry*]Sal, is sufficient to eliminate or at least greatly reduce the level of sn^w destabilizing activity.

Complementation tests between pairs of mutant P *elements fail to restore the* sn^w *destabilizing activity*

The four open reading frames of the non-defective 2.9 kilobase P element could encode several different polypeptides, or could be spliced together by RNA processing to produce a single protein. If the activity responsible for destabilizing sn^w resides in a single polypeptide, then the four different frameshift lesions in the four ORFs would all be affecting the same molecule, and no complementation would be expected between pairs of mutants. If, on the other hand, there are two or more different subunits encoded by the ORFs, then the presence of two different mutant P elements within the same genome should restore some level of enzyme activity. That is, some combination of mutant elements should induce the destabilization of sn^w, where neither element alone could do so.

By the appropriate genetic crosses, we generated flies carrying all six pairwise combinations of the four frameshift mutants of Pc[*ry*], and tested the stability of the sn^w allele in these lines. Some 2000 gametes bearing sn^w were scored for each pair, but in no case was any evidence found for destabilization of sn^w (table 2). This strongly suggests that all four ORFs contribute to a single polypeptide involved in the destabilization of sn^w.

Transcripts of P *in flies containing* Pc[*ry*] *are a subset of those found in natural* P *strains*

Poly(A)$^+$ RNA from dysgenic (P male × M female) embryos and from non-dysgenic (P × P) embryos of the P strain two were blotted onto nitrocellulose and hybridized with a number of single-strand probes derived from M13 subclones of the 2.9 kilobase P element. (See figure 2, lanes 1 and 2.) A complex pattern of bands, ranging in size from 0.5 kilobases to greater than 4 kilobases appeared when the blots were probed with fragments from either the left- or right-hand side of the element. The most prominent species were 2.5, 1.4, 0.9 and in some preparations 0.55 kilobases. Probes derived from more internal segments of the 2.9 kilobase element hybridized primarily to the 2.5 kilobase transcript, but not to the majority of the smaller transcripts (not shown). It is likely that the small RNAs are transcribed from internally deleted P elements (see discussion section). There are no obvious differences in the transcriptional pattern of P elements in the quiescent (P × P) and active (P male × M female) states.

In contrast, the pattern of transcripts from two strains carrying the Pc[*ry*] autonomous element was relatively simple (figure 2, lanes 3–11). The only detectable bands corresponded to the 2.5 kilobase major transcript, and a 3.0 kilobase minor transcript found also in the authentic P strain RNA. The transcripts were not detected by a probe hybridizing to the first 52 bases of the P element (lane 5), nor to a probe derived from the flanking sequences carrying the *rosy* gene (lane 12). No transcripts were found when probes from the opposite strand of the P element were hybridized to the blots (lanes 13–15).

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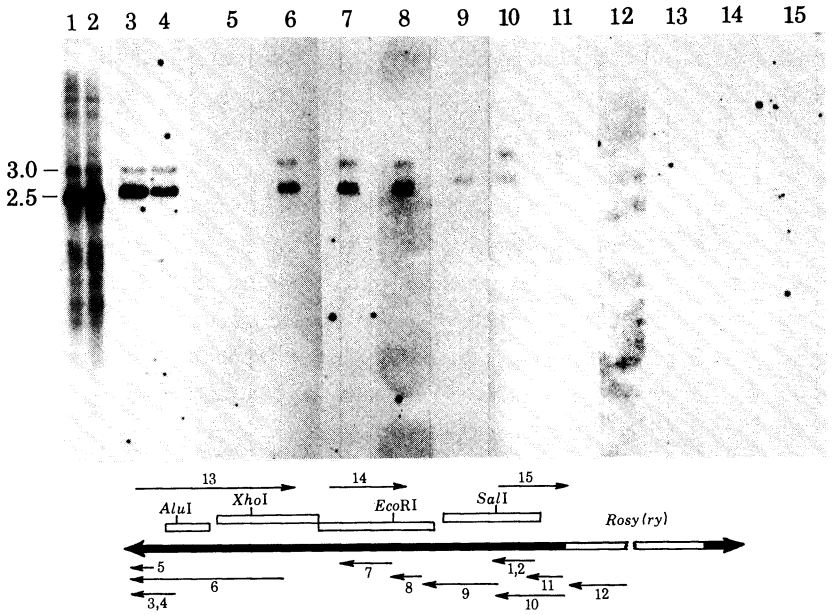

FIGURE 2. RNA blot hybridization to P element transcripts. Lanes 1 and 2 have embryonic poly(A)$^+$ RNA from, respectively, $\pi 2$ (P) male × M female dysgenic hybrid embryos, and $\pi 2 \times \pi 2$ non-dysgenic embryos. Lane 3, RNA from line Pc[*ry*].2. Lanes 4–15, RNA from line Pc[*ry*].17c. The location of radiolabelled single stranded probes relative to the map of Pc[*ry*] are indicated below. The probes are named by a number corresponding to the lane in which they were used. The arrows on the probes indicate their 5′ to 3′ orientation.

DISCUSSION

We have shown by a number of criteria that Pc[*ry*] behaves as a wild type, non-defective P element. It autonomously transposes upon injection into an M strain embryo; it continues to excise and transpose within the resident genome as long as it is maintained in the M cytotype; it destabilizes the hypersensitive dysgenically-induced mutation *singed-weak* (*sn*^{w}).

Mutagenizing the Pc[*ry*] element in any one of the four open reading frames of the P sequence eliminates the transposase activity associated with the element, but does not affect signals required in *cis* for transposition. Taken together with our failure to observe complementation between mutations in different ORFs, the results suggest that each of the four ORFs contributes to a single polypeptide having transposase activity. That we find long RNA transcripts embracing the greater portion of all four reading frames (figure 2) is consistent with this view.

If all four ORFs contribute to transposase, where will the cytotype determining factor be encoded? Assuming cytotype is determined by a protein (e.g. a repressor; see Engels 1981; O'Hare & Rubin 1983) one might expect it to recognize some of the same DNA sequences as does the transposase, for example the inverted repeats. If so, a protein structure that shares certain domains with the transposase would not be surprising. Conceivably, differential RNA transcription or splicing patterns could generate both transposase and repressor from different portions of the same ORFs. The two transcripts we detected may correspond to transposase and repressor message.

The two wild-type Pc[*ry*] transformed lines whose poly(A)+ RNA was examined showed the same two transcripts of 2.5 and 3.0 kilobases, which co-migrated with transcripts found in natural P strains. The other smaller RNA species observed in the natural P strains, some of which are quite prominent, do not appear at all in the synthetic strains, suggesting that they

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are indeed transcripts of defective elements, and not relevant to the expression of hybrid dysgenesis. That transcripts exist covering the greater portion of all four reading frames is consistent with the mutant analysis, and is good circumstantial evidence that one of the two species encodes the transposase activity. We have preliminary evidence from S1 nuclease analysis that splices do connect at least ORFs 1 and 2.

We examined the embryonic RNA of dysgenic hybrids because genetic evidence indicated that hybrid dysgenesis does occur at this stage (see Engels 1983). We detected no reproducible differences between the transcriptional patterns of P elements in dysgenic and non-dysgenic embryos. This was somewhat surprising in that the simplest class of models predicts that the transposase message will be present only in the dysgenic hybrids, and the transcript for the cytotype determining factor will be expressed only in the P strain embryos. Unfortunately, as the Pc[*ry*]-transformed lines all were functionally dysgenic, we were unable to compare the transcriptional pattern of the element in the quiescent (P) and active (M) states. Further work will be needed to elucidate the molecular basis of cytotype determination.

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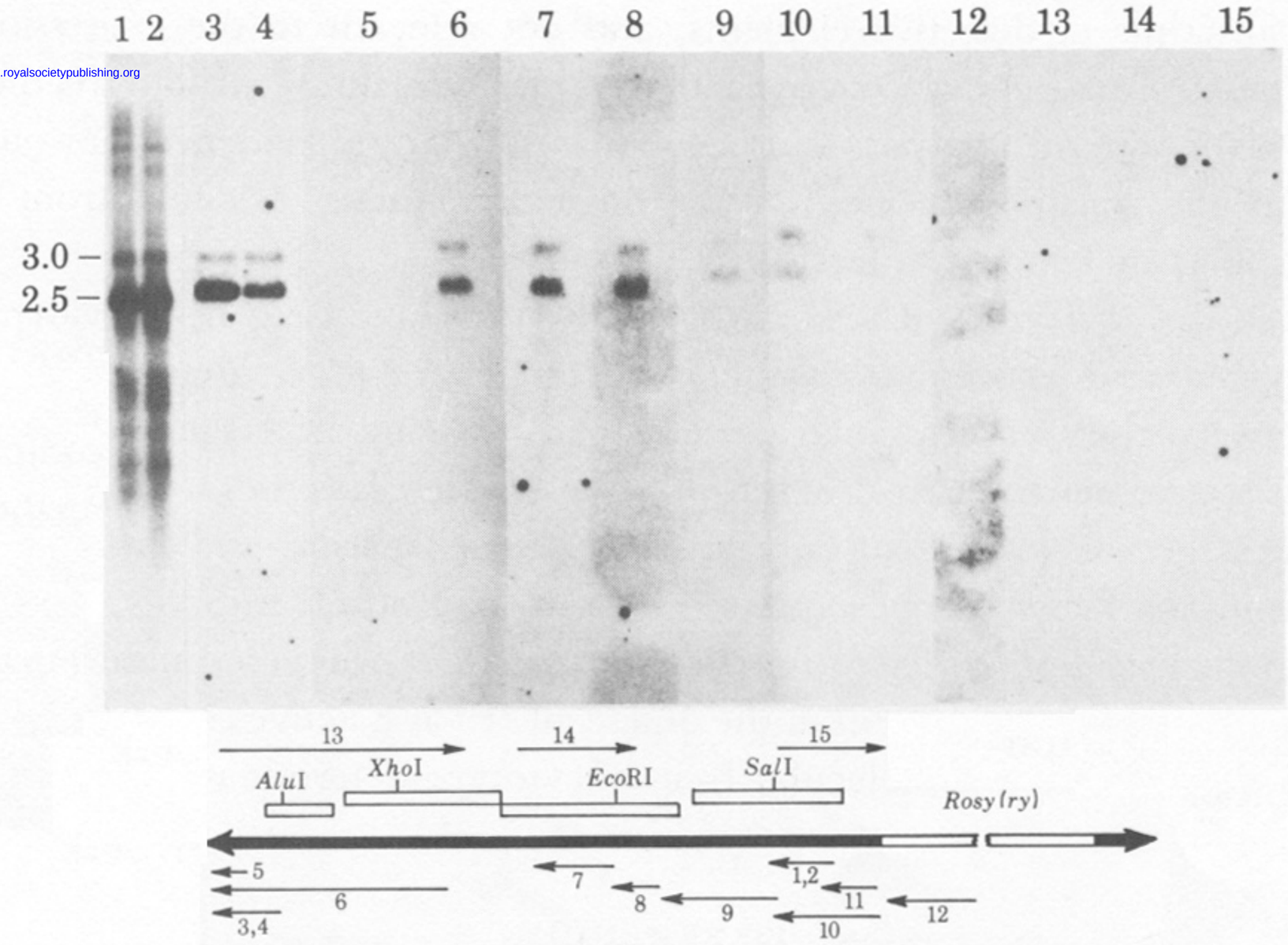

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