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Distribution, inheritance and linkage relationships of ribosomal DNA spacer length variants in pea

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Summary. DNA restriction endonuclease fragment analysis is used to examine the genetic organization, inheritance and linkage associations of the ribosomal DNA in pea. The substantial variation observed in the length of the intergenic spacer region is shown to segregate in Mendelian fashion involving two independent genetic loci, designated *Rrn1* and *Rrn2*. Linkage between *Rrn1* and two marker loci on chromosome 4 establishes the approximate location of this tandem array. *Rrn2* shows linkage with a set of isozyme loci which assort independently of other markers on all seven chromosomes. Combining these observations with previous cytological data, we suggest that *Rrn2* and the isozyme loci linked to it constitute a new linkage group on chromosome 7. The general absence of spacer length classes common to both rRNA loci in any of the lines we examined indicates that little or no genetic exchange occurs between the nonhomologous nucleolar organizer regions.

Key words: Ribosomal DNA spacer length – Restriction fragment length variation – Intrachromosomal mapping – Nucleolar evolution – *Pisum sativum* L.

Introduction

The ribosomal DNA of higher plants is organized as arrays of tandemly arranged repeats, each of which contains both a ribosomal RNA transcription unit and an intergenic spacer (IGS) sequence (Long and Dawid 1980, for a review). The family of rRNA coding sequences contained within these arrays is generally highly conserved, whereas the spacer regions often exhibit extensive intraspecific variability in both sequence and length (e.g., Saghai-Maroof et al. 1984; Jorgensen et al. 1982; Appels and Dvorak 1982; Appels et al. 1980; Gerlach and Bedbrook 1979; Delseny et al. 1979).

Spacer length variation usually results from changes in the copy number of small tandem subrepeats which are located within the IGS and which display little sequence heterogeneity. Variants can be detected on Southern blots as bands that differ from one another in length by an integral multiple of the subrepeat length. In the common garden pea, *Pisum sativum*, which contains approximately 4,000 rDNA tandem repeats (Ingle et al. 1975), restriction analysis has shown that spacer length variants differ by multiples of approximately 180 bp (Jorgensen et al. 1982 and unpublished results). Variants differ by 115 bp in both barley, *Hordeum vulgare* (Saghai-Maroof et al. 1984) and oat, *Avena barbata* (P. D. Cluster, unpublished), by 130 bp in wheat, *Triticum* spp (Appels and Dvorak 1982) and by 325–334 bp in broadbean, *Vicia faba* (Yakura et al. 1984; Lamppa et al. 1984).

The genetic organization and inheritance of ribosomal DNA have been studied in *Xenopus laevis* (Reeder et al. 1976), mouse, *Mus* spp (Arnheim et al. 1982), *Drosophila melanogaster* (Boncinelli et al. 1983), pea (Ellis et al. 1984), barley (Saghai-Maroof et al. 1984), wheat (Snape et al. 1985) and oat (P. D. Cluster, unpublished) using spacer length variants as genetic markers. In each case the observed variation was stable across generations and inherited in a simple Mendelian fashion. The nonrandom distribution of spacer length variants across loci indicates that genetic exchange

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among nucleolar organizer regions located on non-homologous chromosomes is normally limited (see, e.g., Arnheim et al. 1982); although, the occurrence of some variants at more than one locus in primates (Arnheim et al. 1980; Krystal et al. 1981) and in *Drosophila melanogaster* (Coen and Dover 1983) suggests that non-homologous exchange may occur in some instances.

In this study, the variation in pea ribosomal DNA spacer length is examined by restriction endonuclease fragment analysis and evidence is presented that the fragment patterns observed are specified by two independent genetic loci, the locations of which are described in terms of their linkage relationships with selected genetic markers. It is further demonstrated that little or no genetic exchange occurs between the loci.

Materials and methods

Seed material for pea inbred lines was obtained through the courtesy of Dr. G. A. Marx (NYS Agr. Exp. Sta., Geneva, NY) and designated 1–12 as follows: (1) A778-26-6, (2) A73-91, (3) A1179-395, (4) B980-686, (5) C879-344, (6) A1078-236, (7) A78-237, (8) C482-236, (9) A578-238, (10) A780-388, (11) A1078-234, (12) A583-139.

Isozyme and morphological characters for the F_2 progeny of the crosses 2×7 and 5×2 were scored as described by Polans et al. (1985). Several of these markers and similar characters scored in the F_2 progeny of the crosses 6×9 and 1×12 are listed in both the text and Table 2.

DNAs extracted from the leaves of individual pea plants were digested with restriction endonucleases, fractionated on agarose gels and transferred to Gene-Screen membranes (New England Nuclear) as described by Polans et al. (1985). Ribosomal RNA coding and non-coding sequences were then localized by hybridization with a nick-translated pea genomic clone, pHA2, which contains a complete rDNA repeat unit from *Pisum sativum* L. cv. 'Alaska' (Fig. 1, Jorgensen et al. 1982; Cuellar 1982). Originally cloned into the vector pACYC184 and designated pHA1 by R. E. Cuellar, the repeat unit was recloned into pBR322 and redesignated pHA2 by L. S. Kaufman in this laboratory. Linkage relationships were estimated using the LINKAGE-1 computer program (Suiter et al. 1983).

Results

Pea inbred lines known to vary for a large number of morphological and isozyme characters were surveyed for rDNA restriction fragment length variation. Identification of the degree and pattern of restriction fragment length polymorphism found across the lines led to the construction of crosses that encompassed the range of available variation. Segregation analysis was then performed on the rDNA restriction fragment variants to characterize the genetics of the band pattern phenotypes involved in each cross. The newly-characterized genetic loci were subsequently examined for linkage with segregating genetic markers.

DNA samples from the inbred lines showed substantial rDNA spacer length polymorphism upon digestion with Eco RI (Fig. 2), Bam HI and Hind III. Since the same grouping of lines into five different band patterns was obtained with each of these enzymes, it is unlikely that methylation polymorphism contributed to the variability generated with their use. (One of the four Bam HI sites in cloned pea rDNA shows incomplete cleavage in genomic DNA which may reflect methylation of this site in a portion of the rDNA repeats (Jorgensen et al. 1982). There is no evidence for incomplete cleavage of pea rDNA by either Eco RI or Hind III.) The 3.7 kbp Eco RI band seen in all the lanes of Fig. 2 contains only sequences from the highly conserved rRNA coding region (fragment A in Fig. 1), while the remaining bands represent fragments including the IGS region (fragment B in Fig. 1). These bands vary in length by multiples of approximately 180 bp, the length of the short tandem subrepeats located within the IGS (Jorgensen et al. 1982), and we therefore interpret the observed variation as reflecting variation in the number of subrepeats per spacer fragment.

An inspection of the arrangements of the spacer length variants pictured in Fig. 2 suggested the involvement of two genetic loci, one producing the 4.6 and 4.8 kbp bands (also represented by a and b in Fig. 3) and the upper (5.5 kbp) band in lane 1 and the other producing the remaining higher molecular weight bands (also represented by c and d in Fig. 3). The "allele" or haplotype producing d specifies a group of approximately six length-variable bands, one of which is also 5.5 kbp in length but which otherwise appears to be distinct from the 5.5 kbp band in lane 1 (see below).

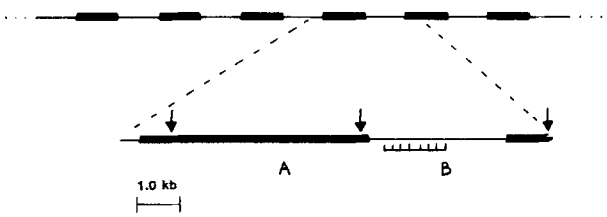

Fig. 1. Schematic diagram of a pea rDNA repeat unit. Derived from the map of pea rDNA clone, pHA1 (Jorgensen et al. 1982). Arrows indicate the sites of Eco RI cleavage that divide the repeat unit into two fragments. Fragment "A" is contained completely within the rRNA transcription unit (*boldface line*) and corresponds to the approximately 3.7 kbp band found in all the pea accessions examined. Fragment "B" includes the intergenic spacer (*thin line*); it is the source of spacer length variation. This variation is generally a result of differences in the number of small tandem subrepeats located within the IGS, represented here by a bracketed region divided into seven sections. Variants observed in this study correspond in length to repeating units containing approximately 5 to 35 subrepeats

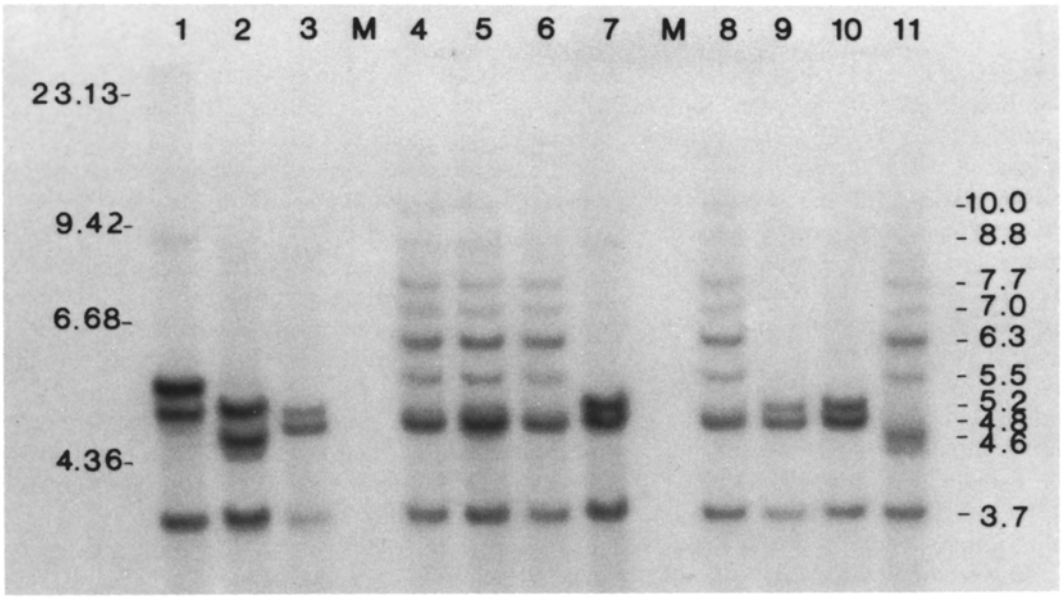

Fig. 2. Variation in pea ribosomal DNA spacer length fragments. DNAs were extracted from the leaves of single pea plants, digested with Eco RI and then probed with pHA2. Lanes marked 1–11 contain DNA from inbred lines 1–11 described in "Materials and methods". *Lane M* contains Hind III-cut lambda DNA size standards (not visible) whose fragment lengths in kilobases are indicated on the *left-hand side* of the figure. The five distinct band patterns that can be discerned are represented by (a) *lane 1,* (b) *lane 2,* (c) *lanes 3, 7, 9* and *10,* (d) *lanes 4, 5, 6* and *8* and (e) *lane 11.* The lengths of the bands comprising these five patterns (with an accuracy of estimation to the nearest 100 bp) are approximately 3.7, 4.6, 4.8, 5.2, 5.5, 6.3, 7.0, 7.7, 8.8 and 10.0 kbp, as indicated on the *right-hand side* of the figure. The 3.7 kbp band is fragment A of Fig. 1, while the other bands correspond to variants of fragment B. The observed length variation is consistent with variation in the number of 180 bp subrepeats, ranging between an estimated 5 subrepeats for the 4.6 kbp band and an estimated 35 subrepeats for the band at 10.0 kbp. Idealized fragment B band lengths in multiples of 180 bp are 4.64, 4.82, 5.18, 5.54, 6.26, 6.98, 7.70, 8.78 and 10.04 kbp, respectively

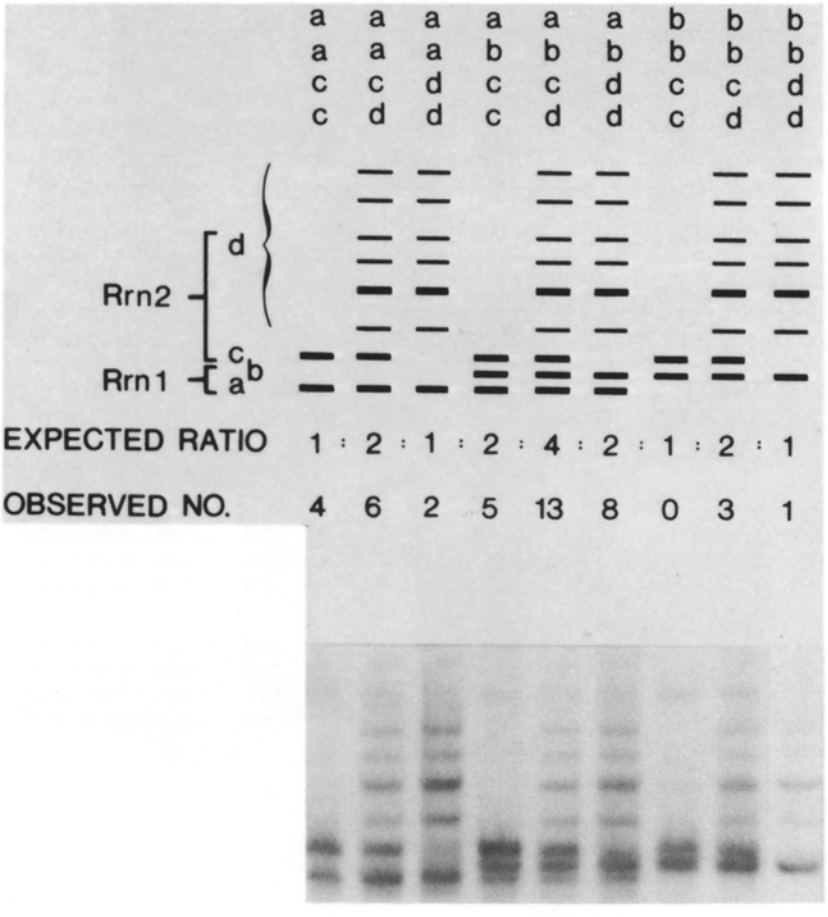

Fig. 3. Segregation and independent assortment of pea ribosomal DNA spacer length variants. DNAs were extracted from the F_2 progeny of the cross 5 (*bbdd*)×2 (*aacc*), digested with Eco RI and then probed with pHA2. The resulting F_2 band patterns reassorted in a manner consistent with segregation at two independent loci (see text). Samples of the F_2 progeny representative of each of the nine phenotypic categories are shown in both autoradiogram and schematic diagram form, along with their observed and expected numbers. DNAs representing the bbcc and bbdd phenotypes were actually taken from pea lines 9 and 6, respectively. The four segregating and assorting band patterns (a–d) are produced by loci *Rrn1* (a, b) and *Rrn2* (c, d) as depicted

Three crosses were selected to test the hypothesis of a two-locus system encoding spacer length variation: 6×9 (Fig. 2; *bbdd*×*bbcc*), 2×7 (Fig. 2; *aacc*×*bbcc*) and 5×2 (Fig. 2; *bbdd*×*aacc*). In the first cross, one putative locus is fixed for b while the second putative locus varies for c and d. In the second cross, c is fixed while a and b vary. In the third cross, lines 5 and 2 vary for both sets of bands.

For the F_2 progeny from the crosses 6×9 and 2×7, each of which varies at only one of the two putative rRNA loci, a comparison of observed and expected numbers of progeny in each phenotypic category showed good agreement with the 1 : 2 : 1 Mendelian ratio for segregation at a single locus, as indicated by χ^2 goodness-of-fit tests (Table 1). There was no observable reassortment of band pattern phenotypes in these crosses. However, F_2 progeny from the cross 5×2, in which the parents differ at both putative loci, did exhibit reassortment of parental band patterns. A comparison of observed and expected numbers of progeny in each of these phenotypic categories showed good agreement with the 1 : 2 : 1 : 2 : 4 : 2 : 1 : 2 : 1 Mendelian ratio for segregation at two independent loci, as indicated by both χ^2 goodness-of-fit and χ^2 contingency tests; $\chi^2_{(8)} = 7.63$, $0.25 < P < 0.50$ N.S. and $\chi^2_{(4)} = 2.78$, $0.50 < P < 0.75$ N.S., respectively (Fig. 3). Analyses of the F_2 progeny from this same cross for single-locus segregation at each of the two loci were also consistent with Mendelian expectations (Table 1). Thus, all the observed variation in pea rDNA spacer length can be explained by genetic control at two independent loci, here designated *Rrn1* and *Rrn2* as shown in Fig. 3.

Because they behave as single loci, both rDNA tandem arrays should map onto the genome as discrete, independent units. Accordingly, linkage relationships between them and an array of other genetic markers were examined. Twelve markers segregated in the F_2 progeny from the cross 6×9, 17 markers segregated in

Table 1. Numbers of observed (and expected) F_2 progeny in three hybrid crosses and associated single locus χ^2 goodness-of-fit analysis

Crosses	Single locus genotype						No. of progeny	Goodness-of-fit 1:2:1 ratio $\chi^2_{(2)}$	
	aa	*ab*	*bb*	*cc*	*cd*	*dd*			
6×9				17 (14.25)	30 (28.5)	10 (14.25)	57	1.88 $0.25<P<0.50$	N.S.
2×7	8 (7.0)	15 (14.0)	5 (7.0)				28	0.79 $0.50<P<0.75$	N.S.
5×2	12 (10.5)	26 (21.0)	4 (10.5)				42	5.42 $0.05<P<0.10$	N.S.
				9 (10.5)	22 (21.0)	11 (10.5)	42	0.28 $0.75<P<0.90$	N.S.

Table 2. Joint segregation analysis involving *Rrn1*, *Rrn2* and associated marker loci

Loci	F_2 progeny in each genotypic class[a], no.									N[b]	χ^2[c]	Recombinant[c]	
	11, 11	11, 12	11, 22	12, 11	12, 12	12, 22	22, 11	22, 12	22, 22			P[c]	fraction
					2×7								
Rrn1, Fa	2		3[d]	0		12[d]	0		4[d]	21	7.1	<0.05	0.18±0.09
					1×12								
Rrn1, Was	5[d]		0	7[d]		0	1[d]		6	19	15.0	<0.01	0.05±0.05
					6×9								
Rrn2, 6pgd-p	7	2	0	1	22	1	0	3	14	50	62.0	<0.01	0.07±0.03
Rrn2, Pgm-c	6	3	0	2	14	3	1	4	9	42	24.8	<0.01	0.18±0.05
6pgd-p, Pgm-c	9	1	0	4	21	2	0	3	11	51	52.4	<0.01	0.10±0.03
					5×2								
Rrn2, Pep-3	5	2	1	2	12	3	0	4	8	37	20.4	<0.01	0.19±0.05
Rrn2, 6pgd-p	5	3	1	4	16	2	0	4	8	43	21.9	<0.01	0.20±0.05
Pep-3, 6pgd-p	5	2	0	1	17	0	0	1	11	37	50.2	<0.01	0.06±0.03

[a] Genotypic designations: 1 = line 1, 2 or 6 haplotype, 2 = line 5, 7, 9 or 12 haplotype
[b] Number of progeny examined
[c] Calculated using the LINKAGE-1 computer program (Suiter et al. 1983, see text)
Expected ratios: 2×7 = 1:3:2:6:1:3, 1×12 = 3:1:6:2:3:1, 6×9 and 5×2 = 1:2:1:2:4:2:1:2:1
[d] Heterozygous phenotype cannot be differentiated due to dominance

the F_2 progeny from the cross 2×7 and 21 markers segregated in the F_2 progeny from the cross 5×2.

Based upon the cross 2×7, linkage was established between *Rrn1* (Fig. 3) and *Fa,* the locus that controls the fasciated stem phenotype, previously assigned to chromosome 4 (Blixt 1974). According to these data, they are separated by 18±9 map units (Table 2). However, the *fa* phenotype was not clearly expressed and a similar association between *Rrn1* and *Fa* could not be established in the cross 5×2, rendering the linkage inconclusive. To resolve the ambiguity surrounding the placement of *Rrn1* on chromosome 4, F_2 progeny were examined from the cross 1×12 which segregates for *Was* and *Td,* markers located on opposite sides of *Fa* (Blixt 1974). Eighteen of the 19 progeny scored were found to be nonrecombinant for *Rrn1* and *Was* (Table 2), indicating tight linkage between these two loci and confirming the presence of *Rrn1* on chromosome 4. Joint segregation ratios for *Rrn1* and *Td* did not deviate significantly from random assortment. The results of this cross also suggest that the 5.5 kbp band found in line 1 (lane 1, Fig. 2) is specified by *Rrn1* and that it assorts independently of the 5.5 kbp band specified by *Rrn2* in d.

Based upon the cross 6×9, *Rrn2* was linked to genes encoding both the plastid isozyme of 6-phosphogluconate dehydrogenase (*6pgd-p*) and the cytosolic isozyme of phosphoglucomutase (*Pgm-c*), mapping 7±3 map units from the former and 18±5 map units from the latter. Consistent with these findings, *6pgd-p* and *Pgm-c* were estimated to lie 10±3 map units from each other, suggesting that *Rrn2* maps on the side of *6pgd-p* opposite *Pgm-c.* The chromosomal locations of both *6pgd-p* and *Pgm-c* are unknown. Based upon the cross 5×2, *Rrn2* was also tightly linked with the genes encoding peptidase-3 (*Pep-3*) and, once again, *6pgd-p,* mapping 19±5 map units from the former and 20±5 map units from the latter. *Pep-3* and *6pgd-p* were located 6±3 map units apart; the chromosomal location of *Pep-3* is also unknown. These data, summarized in Table 2, suggest the following tentative linkage arrangement: *Rrn2,* (*Pep-3, 6pgd-p*), *Pgm-c.* The chromosomal location of this linkage group is discussed below.

The rest of the segregating markers assorted independently of *Rrn1* and *Rrn2* and showed expected linkage relationships among themselves.

Discussion

The two genetic mechanisms of homogenization most often associated with concerted evolution are unequal crossing over and gene conversion. Only unequal crossing over, however, can alter the copy number of a family of genes or, in the case of rDNA, the copy number of a family of subrepeats within the IGS (see Treco et al. 1982, for a review). Unequal exchange at the region of repetition in the IGS (i.e. the subrepeats) would generate length heterogeneity, while unequal exchange at the level of the rDNA repeating units would affect the copy number of the different IGS length variants in an array (Coen et al. 1982). Continued unequal exchange, in addition to altering both spacer lengths and the copy number of these lengths, has been shown to lead to the fixation of sequences within a species (Coen et al. 1982).

We have detected five different rDNA spacer length band patterns characterizing the *Pisum sativum* experimental lines examined (Fig. 2). The bands displayed vary in length by multiples of approximately 180 bp, suggesting that differences in copy number of the 180 bp tandem subrepeats within the IGS are primarily responsible for the observed variation. Genetic analysis shows that these phenotypes are specified by two independent genetic loci. The *Rrn1* locus, controlling the 4.6 and 4.8 kbp bands (also see a and b, Fig. 3) and the 5.5 kbp band in lane 1 (Fig. 2), shows no length heterogeneity within any individual rDNA array; within our limits of detection, only a single spacer length class is displayed in each case. In contrast, the *Rrn2* locus, controlling the remaining higher molecular weight bands, can be either homogeneous (c) or heterogeneous (d) in spacer length. The high variability in spacer length within these latter rDNA arrays may reflect the effects of unequal exchange leading to the fixation of variant IGS sequences (Dover 1982). A similar observation of homogeneity within the shorter rDNA length classes and heterogeneity within the longer rDNA length classes of pea is reported by Ellis et al. (1984).

We do not know if these differences between *Rrn1* and *Rrn2* are associated in any direct way with the function of ribosomal RNA genes. However, it is interesting to note that recent studies have shown that there is less cytosine methylation in the DNA of the shorter of the two major spacer length variants in the pea cultivar "Alaska" and that changes in the level of methylation occur during development, again primarily in the shorter spacer length class (J. C. Watson, unpublished). Related studies on DNase I hypersensitivity in ribosomal gene chromatin also have indicated the presence of sites distinguishing the shorter and longer forms in light-grown versus dark-grown plants (L. S. Kaufman, unpublished). Taken together, these data are consistent with different patterns of regulation for the two major spacer length variants of cultivar Alaska. Thus the possibility exists that the greater variability and/or generally longer spacer length at *Rrn2* could somehow be connected with a difference in function between the two ribosomal gene loci.

The apparent absence of bands shared by both rRNA loci in any individual or line suggests that little or no genetic exchange occurs between the rDNA tandem arrays located on nonhomologous chromosomes (also see Ellis et al. 1984). Only a 5.5 kbp fragment, produced by *Rrn1* in line 1 (Fig. 2) and by *Rrn2* as the lowest molecular weight band in d (Figs. 2 and 3), is common to both loci over the range of pea lines we have examined; and these may in fact vary considerably in sequence, sharing fragment length by coincidence only. This pattern of spacer length distribution, which further implies the independent molecular evolution of the pea nucleolar organizer regions, parallels more closely the situation found in mouse than that found in humans and apes (Krystal et al. 1981).

Using silver-staining techniques, Lamm (1981) has established the location of nucleolar organizer regions on pea chromosomes 4 and 7, which is consistent with the association found in this study between *Rrn1* and both *Fa* and *Was* on chromosome 4. At present, however, *Rrn2* cannot be definitively assigned to any chromosome. Both *Rrn2* and the isozyme loci linked to it, particularly *6pgd-p,* have displayed independent assortment with most regions of the pea linkage map (Weeden 1985) and, specifically, with all markers on chromosome 7. If *Rrn2* corresponds to the nucleolar organizer region on chromosome 7, it may be isolated at the end of one of the arms and, thus, may be too far from any of the standard markers on chromosome 7 (e.g. *Wsp, R, Tl,* or *Bt*) to exhibit linkage. Alternatively, Lamm (1951) and Folkeson (1984) have suggested that these "chromosome 7" markers may actually be part of chromosome 5. This would explain how *Rrn2, Pep-3, 6pgd-p* and *Pgm-c* might reside on chromosome 7 without showing linkage with any of the linkage group 7 markers. Should the location of linkage group 7 on chromosome 5 be confirmed, then *Rrn2* and its three linked isozyme loci would represent the most appropriate starting point for establishing a new set of marker loci on chromosome 7.

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