The Distribution of Early Recombination Nodules on Zygotene Bivalents From Plants

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ABSTRACT

Early recombination nodules (ENs) are protein complexes ~100 nm in diameter that are associated with forming synaptonemal complexes (SCs) during leptotene and zygotene of meiosis. Although their functions are not yet clear, ENs may have roles in synapsis and recombination. Here we report on the frequency and distribution of ENs in zygotene SC spreads from six plant species that include one lower vascular plant, two dicots, and three monocots. For each species, the number of ENs per unit length is higher for SC segments than for (asynapsed) axial elements (AEs). In addition, EN number is strongly correlated with SC segment length. There are statistically significant differences in EN frequencies on SCs between species, but these differences are not related to genome size, number of chromosomes, or phylogenetic class. There is no difference in the frequency of ENs per unit length of SC from early to late zygotene. The distribution of distances between adjacent ENs on SC segments is random for all six species, but ENs are found at synaptic forks more often than expected for a random distribution of ENs on SCs. From these observations, we conclude that in plants: (1) some ENs bind to AEs prior to synapsis, (2) most ENs bind to forming SCs at synaptic forks, and (3) ENs do not bind to already formed SCs.

DURING prophase I of meiosis, homologous chromosomes align, synapse, and cross over. Some or all of these events may be mediated by recombination nodules (RNs) that are multicomponent, proteinaceous ellipsoids that range from 50 to 200 nm in their longest dimension. RNs are found in association with axial elements (AEs) and synaptonemal complexes (SCs) from leptotene through pachytene (Carpenter 1975; for reviews, see von Wettstein *et al.* 1984; Stack *et al.* 1993; Roeder 1997; Zickler and Kleckner 1998, 1999). Two types of RNs have been identified: early nodules (ENs) and late nodules (LNs), which are differentiated by their time of appearance, size, shape, and relative numbers (Stack and Anderson 1986a,b; Albini and Jones 1987; Carpenter 1987, 1988; Zickler and Kleckner 1999).

While LNs are found at sites of crossing over along SCs during pachytene and probably represent the molecular factories that accomplish crossing over (*e.g.*, Carpenter 1975, 1979; Bernelot-Moens and Moens 1986; Sherman and Stack 1995; Zickler and Kleckner 1999), the role of ENs is less clear. ENs are associated with AEs and SCs from leptotene through early pachytene. ENs are more variable in size and shape than LNs, and ENs are 2 to 20 times more numerous per unit length of SC than LNs (Rasmussen and Holm 1980; Stack and Anderson 1986a,b; Zickler and Kleckner 1999). Sometime during early pachytene, ENs are rapidly lost from SCs to leave only one or a few LNs per bivalent (Stack and Anderson 1986a,b). Although unconfirmed, the current evidence suggests that a subset of ENs become LNs (*e.g.*, Sherman *et al.* 1992; Plug *et al.* 1998; Zickler and Kleckner 1999; Agarwal and Roeder 2000). Because ENs are found associated with axial elements in leptotene before synapsis and because ENs are often present at synaptic initiation sites, ENs may be involved in recognition and synapsis of homologs (*e.g.*, Albini and Jones 1987; Anderson and Stack 1988; Rockmill *et al.* 1995; Zickler and Kleckner 1999). In addition, ENs probably have a role in the early events of recombination since at least some ENs contain the RecA-related proteins, Rad51p and Dmc1p, that are required for DNA homology searching in preparation for crossing over (Anderson *et al.* 1997; Moens *et al.* 1997; Tarsounas *et al.* 1999; Zickler and Kleckner 1999).

In spite of the probable importance of ENs for synapsis and crossing over, there have been relatively few studies of ENs (Rasmussen and Holm 1978; Carpenter 1979; Holm and Rasmussen 1980, 1983; Holm *et al.* 1981; Stack and Anderson 1986b; Albini and Jones 1987). Even so, it is clear that ENs are common in euchromatin, while ENs are relatively rare in heterochromatin. The distribution (spacing) between adjacent ENs in euchromatic regions during zygotene has been reported to be random (Bombyx males, Holm and Rasmussen 1980), nearly random (*Coprinus cinereus*, Holm *et al.* 1981), nonrandom (human, Rasmussen and Holm 1978), or even (Allium species, Albini and Jones 1987; see discussion by Zickler and Kleckner 1999). Given the con-

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flicting reports about EN distribution in different organisms and the growing literature linking specific recombination proteins to ENs, we examined the distribution of ENs on spreads of zygotene SCs from six plant species: two dicots, *Lycopersicon esculentum* (tomato) and *Cyphomandra betacea* (tree tomato); three monocots, *Allium cepa* (onion), *Tradescantia edwardsiana* (spiderwort), and *Lilium longiflorum* (lily); and one lower vascular plant, *Psilotum nudum* (whisk fern). While there were significant differences in EN frequency between the different species, the distribution of EN spacing along SCs in euchromatin was random for each species. In addition, our results indicate that some ENs attach to AEs prior to synapsis, but most ENs attach to forming SCs at synaptic forks where they may temporarily delay the progression of synapsis.

MATERIALS AND METHODS

Plants: All plants used in the study were grown in a temperature-controlled (20°–25°) greenhouse. Data on ENs from *A. cepa* and *C. betacea* were obtained from one plant each, and data from *L. esculentum* (var. Cherry) were obtained from three plants. Data for *P. nudum*, *T. edwardsiana*, and *L. longiforum* were collected from plants in clonal populations that each represent essentially a single plant.

Spreading SCs: The general procedure for spreading SCs was the same for all the species except *L. longiflorum* (see below). Details of the procedures used are described by Stack and Soulliere (1984) for *T. edwardsiana* and *A. cepa*, by Anderson and Stack (1988) for *P. nudum*, and by Sherman and Stack (1995) for *L. esculentum* and *C. betacea*. Briefly, primary microsporocytes at zygotene were placed into a sugar-salt medium and treated with enzymes to remove the cell walls and release protoplasts. The protoplasts were placed onto a plastic-coated slide and exposed either to distilled water or to a dilute solution of nonionic detergent (Nonidet P-40). This caused the cells to swell and burst. Spreads of SCs were dried on the slides before fixing with 4% formaldehyde, followed by staining with uranyl acetate and lead citrate. For *L. longiflorum*, the procedure for obtaining protoplasts was similar, but then the protoplasts were treated with dilute Triton X-100 and centrifuged through a sucrose-step gradient. Aliquots of the nuclear suspension in sucrose were placed onto a thin film of Formvar plastic over an agar surface and fixed on the surface of the agar with 4% formaldehyde (Anderson *et al.* 1997). The plastic was lifted from the agar onto a water surface, grids were placed on the plastic film, and the plastic was transferred onto paper and air dried. Spreads were stained with uranyl acetate and lead citrate.

Microscopy, photography, and measurement: Spreads of zygotene SCs were examined using an AEI 801 electron microscope (EM), and photographs were taken at magnifications ranging from ×4000 to ×10,000.

Zygotene SCs that were largely free from overlying chromatin, nucleolar remnants, and cellular debris and that had clearly visible ENs were selected for analysis. ENs were identified on the basis of their size, shape, staining characteristics, and association with SCs or AEs (Stack *et al.* 1993). Extensive asynapsis, overlapping AEs and SCs, and broken AEs were common in spreads from species with large genomes and long SCs, such as *L. longiflorum*, *A. cepa*, *C. betacea*, *P. nudum*, and *T. edwardsiana*. In these instances, the longest interpretable portions of each bivalent were analyzed. The fraction of synapsis may have been somewhat overestimated in these bivalents because typically the AEs, and not SC segments, were broken. Bivalents with varying amounts of synapsis from the same SC set and bivalents from different SC sets were used. For each species, enough SC length was examined to provide at least 200 ENs for analysis. The only exception to this last criterion was *C. betacea*, for which there were a total of 137 ENs on six interpretable zygotene SCs.

EM negatives were scanned at 300 dpi using an HP ScanJet 4c/T, and the digital images for each SC were assembled into a montage using Adobe Photoshop (version 5.0). The montage was then saved as a bitmap image. A working copy of each SC spread was printed and analyzed to determine the proper tracing of each bivalent and the location of each EN. ENs were identified according to the criteria of Stack *et al.* (1993), using the original EM negatives and a ×8 magnifying loupe (total magnification ~ ×32,000–50,000). The computer program MicroMeasure (Reeves 2001) was used to measure the length of one lateral element (in synapsed regions) and one axial element (in asynapsed regions) of each bivalent from the bitmap image. The locations of ENs as well as synapsed (SC) and asynapsed (AE) segments were recorded during measuring. While only one AE was measured for length, in those rare instances when an EN was observed on the other AE, the approximate position of that EN was also recorded. The data for each SC from each species were compiled into a spreadsheet (Microsoft Excel 97) for summary and analysis.

The frequency of ENs at synaptic forks was also determined. A "fork" was defined as an intersection between synapsis and asynapsis, *i.e.*, the place where central element material ended and where the two axial elements could separate or come closer together because of the absence of transverse fibers and central elements of the SC. The central element was not well preserved in SC spreads from *L. longiflorum*, so we did not use its absence as a criterion for a fork in this species. In this case, paired lengths of AEs that were obviously parallel and separated by ~100 nm were considered to be synapsed. Twists of SCs were not considered to be forks. Only ENs that were exactly at the change from synapsis and asynapsis were counted as being at a fork, so any fork in which the exact position could not be determined (for example, due to a twist in the lateral elements close to or at the fork) was excluded when analyzing EN frequency at forks.

Statistics: The computer program Minitab (version 12.0) was used for most statistical tests. To evaluate whether ENs were randomly distributed along the length of SC segments, we measured the distances between adjacent ENs on uninterrupted SC segments. The distributions of distances between adjacent ENs on SC segments for different species were compared with predicted continuous (normal and gamma) distributions using the Kolmogorov-Smirnov one-sample goodness-of-fit test (Zar 1984; Daniel 1990). Methods of moments estimators were used to generate two parameters for the predicted gamma distributions (β = variance ÷ mean; α = mean ÷ β).

RESULTS

Frequency of ENs per unit length of AEs and SCs during zygotene: Examples of spreads of zygotene SCs with early nodules from each of the six plant species are presented in Figure 1. As noted before for plants (Stack *et al.* 1993), the size of ENs varied within and between species from a minimum of ~50 × 50 nm (*L. esculentum*) to a maximum of ~250 × 290 nm (*C. betacea*). Most of the data used in this study were obtained from middle-to-late zygotene bivalents with ~60–80%

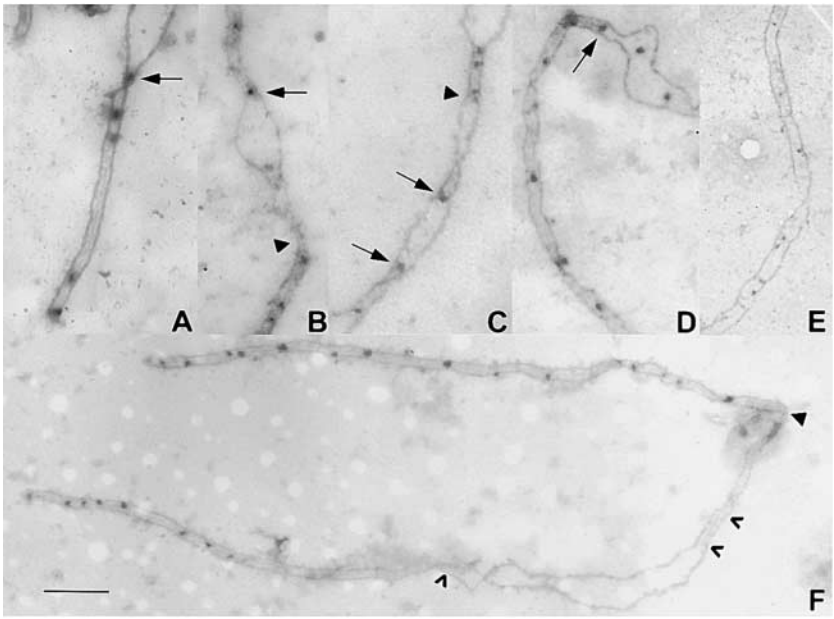

FIGURE 1.—Examples of segments of spread zygotene SCs with early nodules from six different plant species. Arrows indicate some of the synaptic forks with ENs, and carets (^) indicate synaptic forks without ENs. Arrowheads indicate probable synaptic forks that were not used in calculating the frequency of ENs at synaptic forks because the exact location of the fork was questionable. Note that many ENs occur on SC segments while few ENs are found on AE segments. (A) *C. betacea.* (B) *T. edwardsiana.* (C) *P. nudum.* (D) *A. cepa.* In this case, there are two ENs between the AEs, but the AEs do not converge at these positions. (E) *L. longiflorum.* Central elements are not visible in this spread, perhaps due to the use of a different spreading technique (see MATERIALS AND METHODS). (F) A complete zygotene bivalent from *L. esculentum.* Most bivalents from *L. esculentum* are submetacentric to subacrocentric with about one-third of SC length composed of heterochromatin (SHERMAN and STACK 1992). Therefore, although no kinetochore is visible, it is likely that most of the asynapsis in the lower right of the photo runs through heterochromatin. There are no RNs in this region while numerous ENs are visible on the distal, euchromatic segments of the SC. Note the dark telomeres on both ends of the intact bivalent. Bar, 1 μm.

of their length synapsed (Table 1). However, because there was some breakage of axial elements in all species except tomato, the amount of synapsis per bivalent is probably somewhat overestimated. Presumably most of the synapsis was homologous since no partner trades and only a few short regions of foldback synapsis were observed. Because bivalent length and amount of asynapsis per bivalent varied, EN data were expressed as the number of ENs per micrometer of AE or per micrometer of SC. The number of ENs on a pair of AEs that had begun to synapse was determined by measuring the length of one AE but counting ENs on both (presumably) homologous AEs. The number of ENs per micrometer of SC (frequency) ranged from 0.38 for *A. cepa* to 1.61 for *L. longiflorum*, while the number of ENs per micrometer of AE ranged from 0.06 for *A. cepa* to 0.58 for *L. longiflorum* and *T. edwardsiana* (Table 1). Overall, ENs are 2.5–7.5 times more numerous per unit length for SC compared to AE. Because the low frequency of ENs associated with AE could be caused by using mainly middle-to-late zygotene bivalents in which heterochromatic segments (that synapse late and have few ENs) may be overrepresented among remaining AEs, we also determined the frequency of ENs associated with AE for bivalents with <50% synapsis. In this case, the frequency of ENs per micrometer of AE went up for two species, *L. esculentum* (0.22–0.27) and *P. nudum* (0.14–0.19), but there were still at least 5 times more ENs associated with SC than with AE for both species.

Relationship between the number of ENs and SC segment length: The numbers of ENs per SC segment length are plotted for each species in Figure 2, and the summary of the regression equations is presented in Table 2. For each species, there is a strong linear relationship between EN number and SC segment length. The intercept of each regression line is not significantly different from zero (with the exception of *P. nudum*), and the slope of each regression line is positive and

TABLE 1

Frequency of ENs associated with SCs and AEs during zygotene for six plant species

Species	Total no. of bivalents (from no. of sets)	Length (μm)			No. of ENs			No. of ENs/ μm SC (A)	No. of ENs/ μm AE (B)	Ratio (A) to (B)
		SC	AE	Total (% syn)	SC	AE	Total (% on SC)			
A. cepa	3 (1)	536.7	173.3	710.0 (76)	206	10	216 (95)	0.38	0.06	6.3
C. betacae	6 (5)	205.1	117.7	322.8 (64)	123	9	132 (93)	0.60	0.08	7.5
L. esculentum	18 (9)	266.8	163.8	430.6 (62)	375	36	411 (91)	1.41	0.22	6.4
L. longiflorum	3 (1)	127.5	32.9	160.4 (79)	205	19	224 (92)	1.61	0.58	2.8
P. nudum	8 (4)	195.8	127.5	323.3 (61)	204	18	222 (92)	1.04	0.14	7.4
T. edwardsiana	5 (5)	139.2	48.6	187.8 (74)	199	28	227 (88)	1.43	0.58	2.5

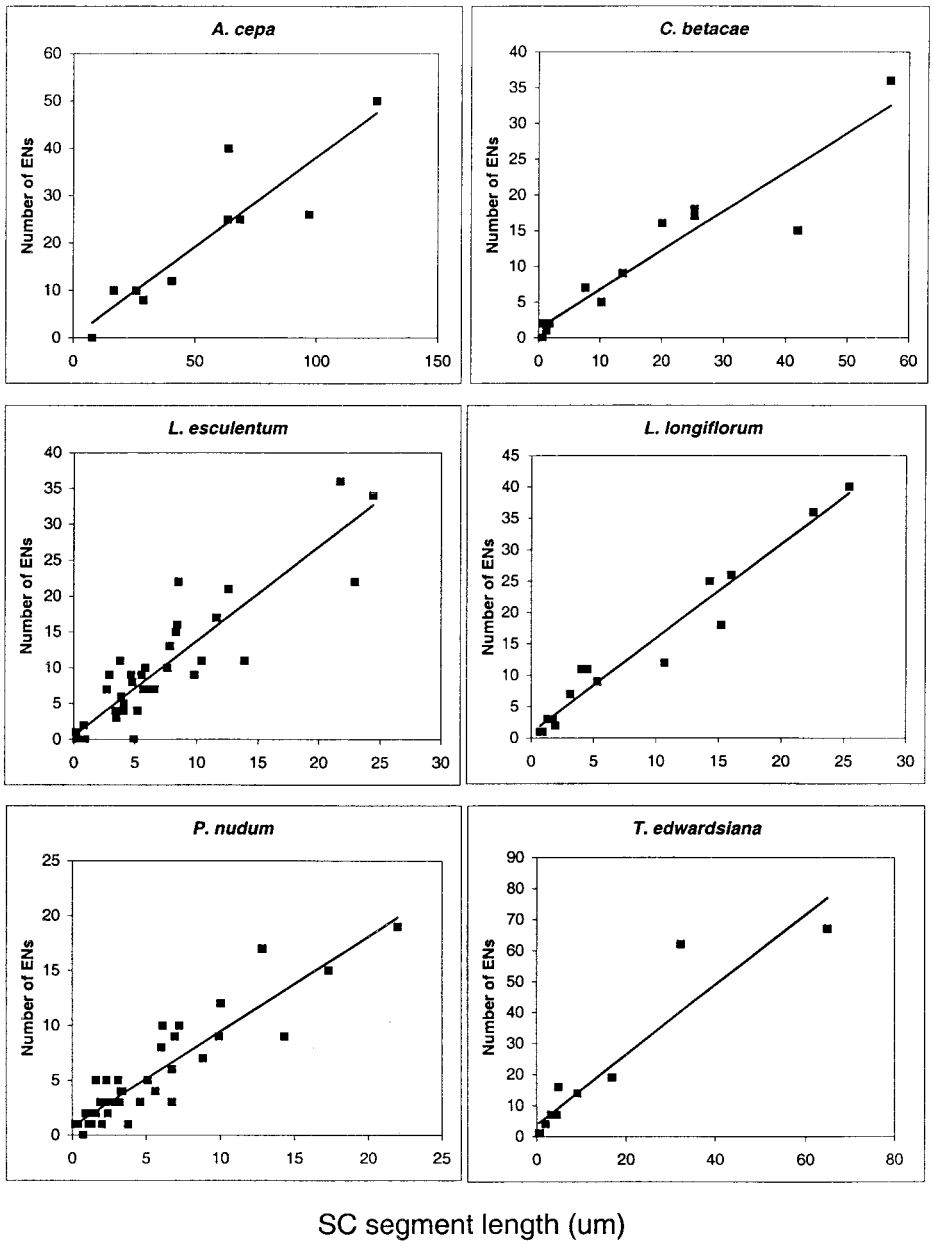

FIGURE 2.—Plots of EN number and SC segment length for six plant species. There is a strong, positive, linear relationship between these two variables for each species. The slopes of the lines differ between species (see Table 2).

significantly different from zero. The slopes of the regression lines range from 0.38 (*A. cepa*) to 1.50 (*L. longiflorum*). These numbers are similar but slightly lower than the frequencies of ENs per micrometer of SC calculated in Table 1. The differences between the slopes are significant ($P < 0.001$; ZAR 1984). Thus, while the frequency of ENs associated with SC segments varies between species, SC segment length is an excellent predictor of EN number for each species ($r^2 = 0.81$–0.95; Table 2).

Relationship between the progress of synapsis and the frequency of ENs: To determine if there is a difference in the number of ENs on SC segments that form early in zygotene compared to SC segments that form late in zygotene, we examined the relationship between the frequency of ENs on SC segments and the percentage of the bivalent length that is synapsed. We assume that bivalents that are earlier in zygotene have a smaller fraction of their length synapsed. The primary analysis was limited to *L. esculentum* in which a bivalent could be verified to be intact by the presence of telomeres at both ends (STACK and ANDERSON 1986a). However, because pericentric heterochromatic regions of *L. esculentum* bivalents synapse late and have few ENs (STACK and ANDERSON 1986a), SCs at later stages of zygotene have an increasing proportion of SC in heterochromatin that could reduce the calculated frequency of ENs on SC segments. Therefore, we used only SC formed in euchromatin [roughly SC in the distal thirds of each chromosome (SHERMAN and STACK 1992)] to calculate the frequency of ENs on SC segments. Eighteen *L. esculentum* bivalents were analyzed that ranged from early

TABLE 2

Summary of regression equations for EN number and SC segment length for six plant species

Species	Slope			Intercept			Reg MS (d.f.)	Res MS (d.f.)	r^2
	Coefficient	SD	*P* value	Coefficient	SD	*P* value			
A. cepa	0.38	0.06	<0.001	0.26	4.18	0.95	1771.1 (1)	52.4 (8)	0.81
C. betacae	0.55	0.06	<0.001	1.32	1.49	0.40	1055.7 (1)	13.3 (10)	0.89
L. esculentum	1.32	0.10	<0.001	0.55	0.83	0.52	2535.0 (1)	13.5 (40)	0.82
L. longiflorum	1.50	0.09	<0.001	0.91	1.07	0.41	2155.4 (1)	8.0 (13)	0.95
P. nudum	0.86	0.06	<0.001	0.83	0.40	0.05	721.8 (1)	3.48 (40)	0.84
T. edwardsiana	1.13	0.14	<0.001	3.79	3.15	0.26	4987.0 (1)	75.1 (9)	0.88

Reg MS, regression mean square; Res MS, residual mean square.

zygotene (14% of length synapsed) to late zygotene (88% of length synapsed). The number of ENs per micrometer of euchromatic SC was plotted against the fractions of bivalent that were synapsed (Figure 3). The slope of the regression is not significantly different from zero ($y = -0.71x + 2.0$, $P = 0.22$, $r^2 = 0.09$), indicating that the frequency of ENs on SC segments does not change from early to late zygotene. From the available data, the other species do not obviously differ from this conclusion for *L. esculentum*.

Frequency of ENs at synaptic forks: A casual examination of zygotene SCs and ENs suggests that ENs are located at synaptic forks more often than would be expected if ENs occur at random along formed SC segments. To investigate this possibility, we examined a total of 140 synaptic forks among the six species. We did not attempt to distinguish whether forks were present in euchromatic or heterochromatic regions. We found that 22–50% of the forks had an EN (Table 3). Assuming that an EN occupies 0.1 μm of SC, we calculated the number of ENs per micrometer of SC at forks and compared that to the observed frequency of ENs along SCs for each species. This comparison shows that ENs are 1.6–12.2 times more common at synaptic forks than along zygotene SCs in general.

Spacing between adjacent ENs: To determine if there is a pattern in the spacing of ENs along SCs, we measured the distance between adjacent ENs for each species. Because the frequency of ENs is low on AEs, we considered only the distances between adjacent ENs on the same synapsed segments. Thus, distances between two ENs on adjacent synapsed and asynapsed segments, distances between adjacent ENs on two SC segments that were separated by an asynapsed segment, and distances between ENs on asynapsed segments were not included in this analysis.

As expected, species with higher frequencies of ENs per micrometer of SC have lower average distances between adjacent nodules, and species with a lower frequency of ENs per micrometer of SC have longer average distances between adjacent ENs (Tables 1 and 4). The minimum distance between two ENs is ~0.1 μm for all six species, but the maximum distance between two ENs varies greatly depending on the species. *L. longiflorum* has the smallest maximum value between two adjacent ENs (3 μm), and *A. cepa* has the largest maximum value (18 μm). The distribution of distances between ENs is presented in a histogram (Figure 4). For each species, the distribution is skewed to the left. Statistical tests confirm that the distributions are not normal (Anderson-Darling normality test, $P < 0.001$ for each species). Therefore we compared the values for the different species using the Mood median test that does not require normal distributions (Table 4). The species fall into three statistically different ($P < 0.001$) categories with regard to median distance between adjacent ENs: (1) *A. cepa* (median = 1.89), (2) *C. betacea* and *P. nudum* (medians = 1.08 and 0.88, respectively), and (3) *L. longiflorum*, *L. esculentum*, and *T. edwardsiana* (medians = 0.46, 0.49, and 0.48, respectively).

Using parameters taken from the observed distributions, the predicted normal and gamma distributions for each species have been plotted for comparison (Fig-

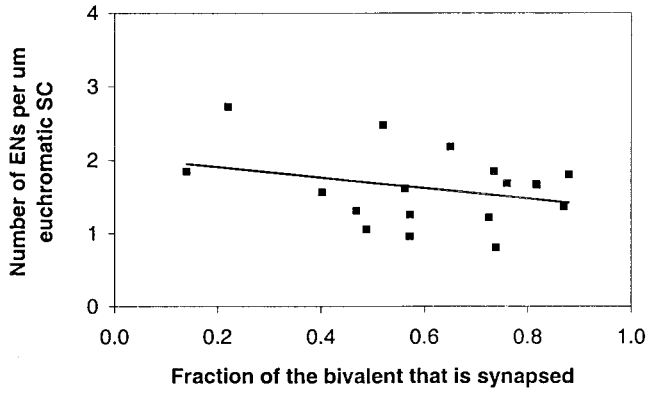

FIGURE 3.—Plot of EN frequency per unit length of SC in euchromatin from *L. esculentum* bivalents and the fraction of the bivalent that is synapsed. The regression equation for the line is $y = 0.72x + 2.0$ ($r^2 = 9\%$). The slope of the line is not significantly different from zero, indicating that the number of ENs per micrometer of SC does not change during zygotene.

TABLE 3

Frequency of ENs at synaptic forks compared to frequency of ENs along AE and SC segments

Species	No. of synaptic forks		No. of ENs/ μm SC at forks[a]	Ratio of EN frequency at forks to EN frequency on	
	With EN (%)	Without EN (%)		AE[b]	SC[b]
A. cepa	6 (46)	7 (54)	4.62	77.0	12.2
C. betacea	4 (50)	4 (50)	5.00	62.5	8.3
L. esculentum	9 (22)	31 (78)	2.25	10.2	1.6
L. longiflorum	5 (42)	7 (58)	4.17	7.2	2.6
P. nudum	27 (50)	27 (50)	5.00	35.7	4.8
T. edwardsiana	5 (38)	8 (62)	3.85	6.6	2.7

[a] The length of SC at a synaptic fork was estimated to be about the size of a nodule that equals 0.1 μm.
[b] From Table 1.

ure 4). Visually, gamma distributions fit the observed distributions much better than normal distributions. These results were confirmed by using the Kolmogorov-Smirnov one-sample goodness-of-fit test (Table 4; Zar 1984; Daniel 1990). As expected, none of the observed distributions fits the normal distributions ($P > 0.05$). However, the distributions for four species (*A. cepa*, *C. betacea*, *L. longiflorum*, and *P. nudum*) are not significantly different from the predicted gamma distributions ($P < 0.05$). The fit between the observed distribution and the predicted gamma distribution for *T. edwardsiana* was marginally rejected at the 0.05 level. However, given that rather rough approximations were made for the parameters of the predicted gamma distribution and that there is only a small difference between the sample D_{max} and the critical value at $\alpha = 0.05$, it is likely that the distribution of distances between ENs for *T. edwardsiana* is also gamma. In contrast, the distribution of distances between ENs for *L. esculentum* is significantly different from a gamma distribution. However, when we consider only SC segments in euchromatin by limiting the data to bivalents in which SC segments comprised 50% or less of the total bivalent length, then the distribution of distances between adjacent ENs is not significantly different from a gamma distribution. Therefore, for each of the six plant species, the observed distributions of distances between ENs are not significantly different from the predicted gamma distributions. We were not able to detect a recurrent distance (or a multiple of a recurrent distance) between adjacent ENs that would imply ENs are laid down in a regular, repeated pattern.

DISCUSSION

The frequency of ENs is higher on SC segments compared to axial element segments: The frequency of ENs associated with SCs ranged from 0.38 to 1.61 ENs/μm of SC for the six plant species (Table 1). These numbers are comparable to those reported by Stack and Anderson (1986b) who observed 1.41 ENs/μm of zygotene SC from *L. esculentum* and by Albini and Jones (1987) who observed 0.53 and 0.78 ENs/μm of zygotene SC from *A. cepa* and *A. fistulosum*, respectively. For the species investigated here, the number of ENs per microme-

TABLE 4

Distance between adjacent RNs on synapsed portions of zygotene SCs

Species	Total no. of distances (μm) observed between adjacent RNs	Mean distance (μm) between all RNs on SC (SD) range/median
A. cepa	196	2.50 (2.25) 0.10–18.00/1.89*
C. betacea	116	1.61 (1.79) 0.17–14.97/1.08†
L. longiflorum	192	0.61 (0.51) 0.06–3.15/0.46‡
L. esculentum	308	0.74 (0.81) 0.08–10.21/0.49‡
P. nudum	163	1.01 (0.73) 0.08–4.80/0.88†
T. edwardsiana	181	0.70 (0.68) 0.08–4.88/0.48‡

Species with statistically different median distances between RNs are indicated with different superscripts (Mood median test; $P < 0.001$), and species with statistically similar median distances are indicated with the same superscripts (Mood median test; $P > 0.05$).

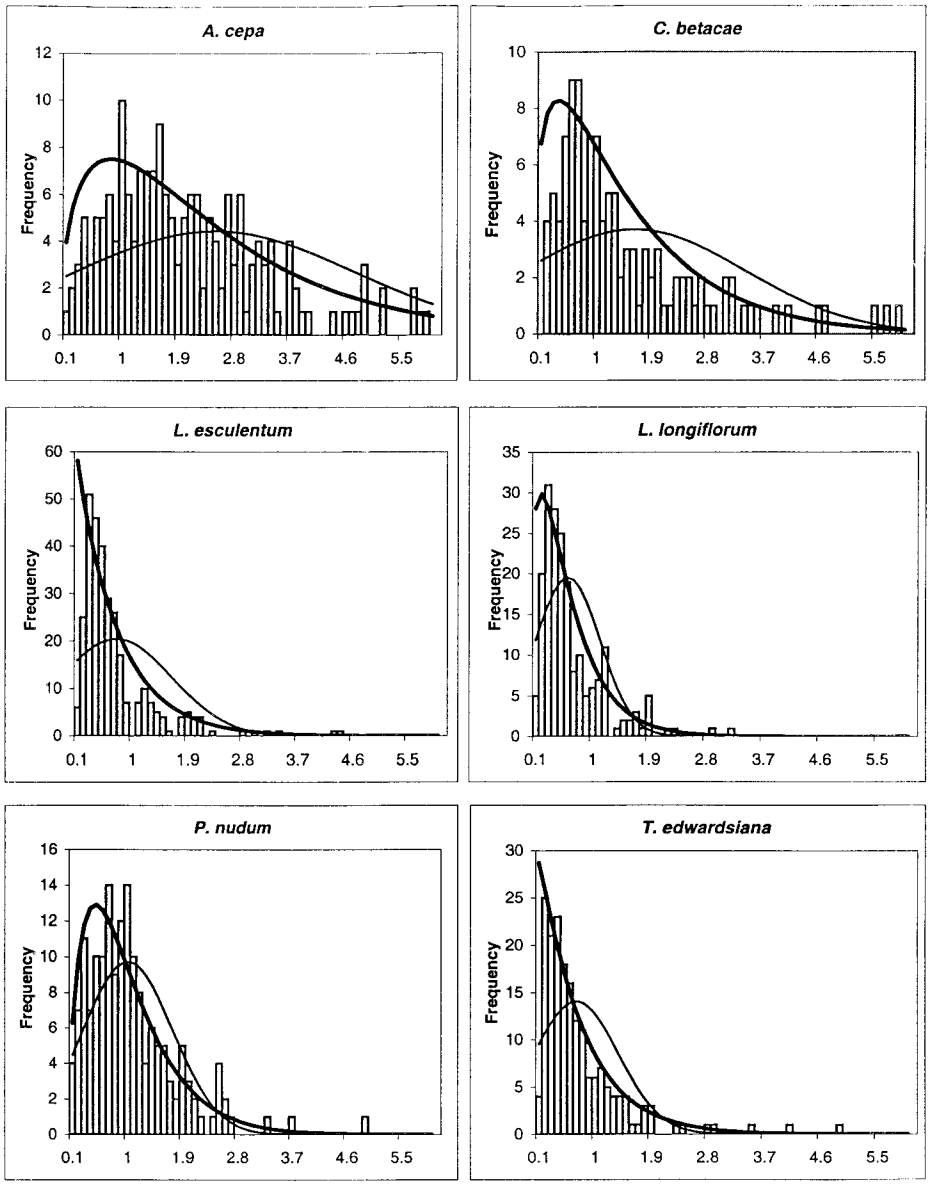

FIGURE 4.—Histograms showing the distribution of distances between adjacent ENs on SC segments for six different plant species. Only internodule distances between ENs on the same SC segment are included. Although the distributions are continuous, they have been presented in 0.1-μm increments. Using parameters estimated from the raw data, a predicted normal curve (thin line) and a predicted gamma curve (thick line) have been superimposed over each distribution. In each case, the predicted gamma curve best fits the observed distributions.

ter of SC was 2.5–7.5 times higher than the number of ENs per micrometer of AE (Table 1). ALBINI and JONES (1987) also found fewer ENs associated with aligned AE than with SC for *A. fistulosum* (0.39 and 0.78, respectively) but approximately the same mean number of ENs associated with both aligned AE and SC for *A. cepa* (0.50 and 0.53, respectively). We were unable to consistently distinguish between aligned and unaligned AEs for the species we examined, and this may be one reason why our value for the number of ENs per micrometer of AE for *A. cepa* is so much lower than that reported by ALBINI and JONES (1987).

What could account for the lower frequency of ENs on AEs compared to SC segments for each species? One possibility is that the frequency is artificially low due to using middle-to-late zygotene bivalents in which a significant proportion of the remaining asynapsed AEs may be located in heterochromatic regions that typically synapse late and have few ENs (*e.g.*, STACK and ANDERSON 1986a; STACK *et al.* 1993). This explanation likely accounts for some of the reduction because when we analyzed zygotene SCs that had 50% or less of their length synapsed, and therefore a larger proportion of axial elements in euchromatin, the frequency of ENs on AEs went up for only two species, *L. esculentum* (0.22 to 0.27) and *P. nudum* (0.14 to 0.19). However, these frequencies were still at least five times lower on AEs compared to SCs. Another possibility is that ENs associated with AEs are less stable during the spreading procedure compared to ENs associated with SCs. To our knowledge, no data that we could use to address this question are available from nondisrupted, serially sectioned nuclei from plants or animals. However, we can test this possibility by comparing our EN frequencies to Rad51p foci frequencies in squashes of *L. longiflorum* nuclei (TERASAWA *et al.* 1995). TERASAWA *et al.* (1995)

found maximal numbers of foci during leptotene (~2000 foci per leptotene nucleus—our calculated number derived from their observations). Assuming that these nuclei have no SC [see comments by Franklin *et al.* (1999) concerning the problem of accurately staging leptotene and zygotene in the absence of an antibody probe for SC components] and assuming 3000 μm of SC for a complete set at pachytene (Anderson *et al.* 1985), then there must be at least twice this length of axial element (2 × 3000 μm = 6000 μm) per leptotene primary microsporocyte nucleus and ~0.33 foci/μm of AE (2000 foci/6000 μm AE). In comparison, we found a frequency of 0.58 ENs/μm AE at zygotene for *L. longiflorum*. Considering the assumptions required to estimate the Rad51p foci frequencies, the probability that two closely adjacent ENs would appear as one fluorescent focus (Freire *et al.* 1998), and the possibility that as many as half of the ENs may not be labeled with antibodies to Rad51p (Anderson *et al.* 1997), these numbers agree reasonably well. This correspondence makes it unlikely that a large proportion of ENs on AEs are being differentially lost during the spreading procedure. Thus, we conclude that the higher frequencies of ENs on SCs compared to AEs probably reflect the situation *in vivo*.

SC segment length is an excellent predictor of EN number: We observed a strong linear relationship (r^2 = 0.81–0.95) between EN number and SC segment length for each species (Figure 2 and Table 2), although there were statistically significant differences between the slopes of the regression lines for the different species. Other investigators have also noted a positive linear relationship between EN number and SC length (human, Rasmussen and Holm 1978; mouse, Moens *et al.* 1997; two Allium species, Albini and Jones 1987; Sordaria, Zickler *et al.* 1992). However, a linear relationship between EN number and SC length does not necessarily mean that ENs are evenly spaced on SCs (see below).

The frequency of ENs per unit length of zygotene SC varies between plant species: We observed up to a fourfold difference in the frequency of ENs per micrometer of SC segment among these six plant species (Tables 1 and 2), and these differences are reflected in the average distance between adjacent ENs on SC segments (Table 4). There is no obvious pattern of EN frequency with regard to phylogenetic class, genome size, or chromosome number. For example, species with higher frequencies of ENs on SCs (>1 EN/μm SC) include the lower vascular plant *P. nudum* ($2n \simeq 200$ and a large genome; S. M. Stack, unpublished observations), the dicot *L. esculentum* ($2n$ = 24; $4C$ = 4 pg), and two monocots, *L. longiflorum* ($2n$ = 24; $4C$ = 141 pg) and *T. edwardsiana* ($2n$ = 12; $4C$ = ~120 pg). The two species with lower frequencies of ENs on SCs (<1 EN/μm SC) include the monocot *A. cepa* ($2n$ = 16; $4C$ = 67 pg) and the dicot *C. betacea* ($2n$ = 24; $4C$ = ~30 pg; DNA contents for all species except *P. nudum* are from Bennett *et al.* 2000). Albini and Jones (1987) also found a difference in EN frequency between two closely related Allium species. We do not know the basis or the possible significance of the differences in EN frequencies on zygotene SCs among plant species. However, it is possible that factors we did not examine such as genotype, ambient temperature, and/or age of plant may have some influence on the observed number of ENs associated with SCs in plants. Almost all of these factors have been shown to affect crossing over in plants (*e.g.*, Griffing and Langridge 1963; Jones 1967; Maguire 1968; Rees and Dale 1974; and see Sherman and Stack 1995 for other references).

Plant ENs attach to AE and forming SC, but not to formed SC: Because ENs are identifiable as discrete structures only by electron microscopy, we often refer to them as if they were preformed complexes that attach to AEs and SC. However, it is more likely that ENs are progressively assembled, probably at sites of DNA double-strand breaks (DSBs). DSBs are required to form Rad51p foci (and probably ENs; Gasior *et al.* 1998; Romanienko and Camerini-Otero 2000), and a number of studies using immunolocalization of two or more proteins have indicated gradual changes in the protein composition of foci during zygotene and pachytene (*e.g.*, Ashley *et al.* 1995; Ashley and Plug 1998; Gasior *et al.* 1998; Plug *et al.* 1998; Agarwal and Roeder 2000; Moens *et al.* 2000; Santucci-Darmanin *et al.* 2000). However, to simplify the discussion and to reflect what we observed microscopically, we refer to binding or attachment of ENs to AEs and SCs with the understanding that ENs are probably assembling at each site.

There are three possible times/locations at which ENs could associate with zygotene bivalents during synapsis: (1) with AEs prior to synapsis, (2) with SC after synapsis, and/or (3) at synaptic forks during synapsis. In regard to the first possibility, we and others (*e.g.*, Albini and Jones 1987) have observed ENs associated with single AEs as well as at convergences of two axial elements (although not all convergence sites have an EN). Convergence sites do not include SC, although SC formation may follow. AE convergences in plants are typically 3–9 μm apart, which represents a frequency of ~0.11–0.33 convergences/μm of SC (see references in Anderson and Stack 1988). This frequency range is similar to the range we observed for ENs associated with AE (0.06–0.58 ENs/μm AE), but lower than the frequency range of ENs associated with SCs (0.38–1.61 ENs/μm SC) for all six plant species (with the possible exception of *A. cepa*; Table 1). Therefore, for most if not all of these plant species, additional ENs must bind to SCs to account for the higher frequencies of ENs associated with SCs.

Do ENs bind to already formed SC during zygotene? Rasmussen and Holm (1978) thought so because, in thin sections from human primary spermatocytes, they observed fewer ENs per micrometer of SC at early zy-

gotene compared to late zygotene. However, we found no change in the number of ENs per micrometer of euchromatic SC in *L. esculentum* from early to late zygotene (Figure 4), and our results from the other five species were consistent with this conclusion. Similarly, Albini and Jones (1987) also examined the frequency of ENs at different zygotene substages and found either no difference (*A. fistulosum*) or fewer ENs per micrometer of SC at later stages of zygotene (*A. cepa*). These results indicate that in plants ENs do not continue to bind to formed SC segments.

An alternative explanation for the lower frequency on ENs on AEs compared to SCs is that ENs bind to SCs at synaptic forks. Here we define a synaptic fork as the interface between synapsis (tripartite SC) and asynapsis (two separated axial elements). Rasmussen and Holm (1978) reported ENs at synaptic forks in human spermatocytes, and we observed that up to 50% of synaptic forks from six plant species have an EN at the fork (Table 3). In addition, for each plant species, the frequency of ENs at forks is higher than along AEs (by a factor of ~7–77) and also higher than along SC segments (by a factor of ~2–12). These results indicate that ENs bind to SCs at synaptic forks. However, if ENs assemble at forks without otherwise interfering with the progress of synapsis, one would expect the frequency of ENs at forks to be the same as the frequency of ENs along SC in general. Since this is not the case, synapsis may be delayed temporarily at forks while ENs are assembled. Then it is likely that synapsis would resume and progress until delayed again as another EN assembles, and so on. In this scenario, ENs that bind at synaptic forks would not be necessary to nucleate SC formation. However, this interpretation does not preclude the possibility that ENs at convergence sites are important for synaptic initiation.

The distribution of distances between adjacent ENs on SC segments is random: The continuous distribution of distances between adjacent ENs for each plant species is presented in histogram form in Figure 4. Each distribution is skewed to the left, and the extent of the shift is related to the different frequencies of ENs per micrometer of SC observed for the different plant species (Tables 1 and 4). While most adjacent ENs are <1 μm apart (except for *A. cepa*), a few adjacent ENs are separated by 3–18 μm. The longer distances between adjacent ENs may be due to SC in heterochromatic regions that have few ENs.

Two curves have been fitted to each of the observed distributions in Figure 4, using parameters derived from the data. One curve is a normal distribution based on the mean and standard deviation, and the other curve is a gamma distribution based on α and β parameters. Both normal and gamma distributions are random distributions for continuous variables, but a gamma distribution differs from a normal distribution in that there can be only positive numbers. This means that normal and gamma distributions will look quite different when means are close to zero, but as means become larger, gamma distributions will look more and more like normal distributions. This is similar to the behavior of Poisson distributions that are used for discrete variables. The constraint of only positive values for gamma distributions is expected when measuring distances between two adjacent ENs since this measurement will always yield a positive number. In addition, there is another constraint on the measurement of internodule distances because the minimum measurable distance between two nodules is limited to the center-to-center distance between two touching ENs. This physical limitation in measuring the distance between adjacent nodules accounts for the scarcity of measured distances ≤ 0.1 μm since only a few nodules are smaller than 0.1 μm in size and very few of those are touching (Figure 4). For each species, the predicted gamma distribution fits the data better than the predicted normal distribution. This result is supported by Kolmogorov-Smirnov one-sample goodness-of-fit statistical tests that show that the distribution of distances between ENs in euchromatin is random and best fits a gamma distribution for each plant species. Although we occasionally observed several adjacent ENs (up to five in one case) that appeared to be evenly spaced, we were unable to detect a recurrent separation distance (or multiple of a recurrent distance) between adjacent ENs in any of the six plant species. Therefore, we conclude that in plants, ENs are randomly spaced along SC segments with respect to one another.

The general appearance of the internodule distance distributions for each plant is similar to those of Coprinus, Bombyx, and humans, as shown by Rasmussen and Holm (1978), Holm and Rasmussen (1980, 1983), and Holm *et al.* (1981). These researchers used a computer model to generate an expected random distribution for distances between adjacent ENs. In each case, the observed and expected distributions were similar. The researchers concluded that the observed distributions were random for Bombyx spermatocytes (Holm and Rasmussen 1980), nearly random for *C. cinereus* (Holm *et al.* 1981), and were not random for human spermatocytes (Rasmussen and Holm 1978). However, no statistical tests were used to assess the fit between the observed and expected distributions. Because of the similarity between the distributions these researchers observed and those we observed for plants, we compared their data [taken from Rasmussen and Holm (1978, Figure 43), Holm and Rasmussen (1980, Figure 59), and Holm *et al.* (1981, Figure 20)] to predicted gamma distributions on the basis of parameters estimated from their data and found that they were not significantly different (Kolmogorov-Smirnov one-sample goodness-of-fit test, $P < 0.05$). Therefore, we conclude that distances between ENs in humans and Coprinus as well as Bombyx are most likely random.

Relation of ENs to LNs: Observations that ENs are

randomly distributed in euchromatin do not necessarily imply that crossovers are randomly distributed. Indeed, hot spots for recombination are well documented in a number of different organisms (Lichten and Goldman 1995). However, we do not know the exact relationship of ENs to LNs that lie at crossover sites. The available evidence suggests that some ENs are converted into LNs (*e.g.*, Plug *et al.* 1998; Zickler and Kleckner 1999; Agarwal and Roeder 2000; Moens *et al.* 2000; Santucci-Darmanin *et al.* 2000). However, it is still not clear whether the large numbers of ENs observed in plants indicate a comparable number of DSBs (Franklin *et al.* 1999), whether all ENs are initially the same and then have different fates depending on their locations and/or time of association with the SC (perhaps related to the mysterious force called interference; *e.g.*, Rasmussen and Holm 1980; Stack and Anderson 1986b), or whether there are initially different types of ENs that look morphologically similar but have different molecular components and different activities (*e.g.*, Dresser *et al.* 1997; Pâques and Haber 1999; Allers and Lichten 2001; see discussion by Zalevsky *et al.* 1999).

Model for the distribution of early nodules: Our results suggest the following model for the frequency and distribution of ENs in plants. Many (perhaps all) ENs associated with AEs serve to link axial elements together at convergence sites where SC formation begins. Assuming that these ENs remain after SCs form, they represent only a minority (10–40%) of the ENs that ultimately will be associated with SCs. The majority (60–90%) of ENs associated with SC are added at synaptic forks. The higher frequency of ENs at synaptic forks is most likely due to a delay in the progress of synapsis while ENs assemble. As synapsis progresses, ENs bind randomly at forks over time so that the distribution of spacing between ENs along SCs is described by a gamma distribution. Thus, beginning at synaptic initiation sites, ENs along a segment of SC represent consecutive events in time. In the context of this model, we suggest two different possibilities for EN composition and behavior: There are two types of ENs, one that initially associates with AEs and another that associates with synaptic forks of SCs. This difference in behavior may be based on a difference in composition and function; *e.g.*, the former might function in synaptic initiation while the latter might function in recombination. Alternatively, all ENs begin with the same basic molecular composition, but ENs progressively change over time. This means that ENs along a segment of SC would represent a developmental hierarchy with the more mature nodules lying closer to synaptic initiation sites and the less mature ENs lying closer to synaptic forks. A more mature EN is more likely to achieve a crossover and become a late nodule than a less mature EN. Once a crossover has been achieved, other nearby ENs would not be able to achieve a crossover due to interference, and these ENs would be lost from the SC *en mass* (Stack and Anderson 1986b). Using antibodies to different molecular components of nodules, it should be possible to test which, if either, of these two alternatives describes the situation in plants.

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