MLH1p and MLH3p Localize to Precociously Induced Chiasmata of Okadaic-Acid-Treated Mouse Spermatocytes

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ABSTRACT

With the phosphatase inhibitor, okadaic acid, we induce the precocious onset of the chiasmate stage and under those conditions show that the recombination nodules, MLH1 and MLH3 foci, are localized to the chiasmata. It is concluded that MLH1/3 foci are appropriate markers for the studies of crossovers/chiasmata development and distribution at late meiotic prophase.

From the correlation between crossover distribution and the distribution of synaptonemal complex (SC)-associated dense bodies in oocytes of wild-type and recombination-defective Drosophila melanogaster females, Carpenter (1975, 1979a,b) proposed that these bodies, recombination nodules (RNs), represent protein complexes that function in recombination. In the following decade, observations of plant, fungal, insect, and mammalian meiotic prophase nuclei also showed a positive correlation in general between the distribution of RNs and chiasmata (Zickler 1977; Rasmussen and Holm 1978; Gillies 1979). Chiasmata have been demonstrated to be the sites of reciprocal genetic exchange at prophase of meiosis I in Locusta (Tease and Jones 1978) and in mice (Kanda and Kato 1980). The same was demonstrated for mouse oocyte diakineses and metaphase I bivalents by 5-bromo-2′-deoxyuridine-2-C14 (BrdU) incorporation as shown in Figure 1 (with permission from Polani 1981 and Traut 1991). Particularly convincing for the correlation between chiasmata and RNs were the observations of Allium and Chlorella where the chromosomal positions of the highly localized chiasmata coincide with the positions of the RNs (Albini and Jones 1984; Bernelot Moens and Moens 1986). However, direct proof for the hypothesized functional connection was difficult to obtain since the RNs are usually no longer present at the time that the chiasmata can be observed.

The discovery that MLH1 and MLH3 foci (Escherichia coli MutL DNA repair homologs) of meiotic prophase chromosome cores/SCs correlate with the known frequency and distribution of crossovers/chiasmata in male and female mice and humans (Baker et al. 1996; Lipkin et al. 2002) has permitted a rapid and much more detailed study of crossover events along individual chromosomes (Froenicke et al. 2002) than would have been possible from the laborious and less suitable metaphase I meiotic chromosome spreads. The distribution of MLH1 immunofluorescent foci along the meiotic chromosome cores has recently produced quality information on genetic recombination in male vs. female distribution, the nature of genetic interference, and possible recombinational hot spots (Barlow and Hulten 1998; Anderson et al. 1999; Hassold et al. 2000; Koehler et al. 2002; Lynn et al. 2002).

Although the results of those studies are reliable and convincing, there is uncertainty about the exact relationship among chiasmata/crossovers, RNs, and MLH1/3 foci. The difficulty in the verification of this relationship is that under normal meiotic prophase development, RNs and MLH1/3 foci are observable mostly prior to the formation of mature chiasmata. Occasionally, MLH1/3 immunofluorescent foci have been detected in stretches of synapsed cores of bivalents that have initiated separation of the cores (Baker et al. 1996; Lipkin et al. 2002), suggesting that those foci may eventually be the sites of chiasmata as in Figure 2 (from Figure 6A). We previously provided the immunoelectron microscope evidence that MLH1p is localized to the RNs (Moens et al. 2002) and preliminary electron microscope (EM) evidence that the RN-MLH1 structure is at the site of a chiasma.

To provide formal proof that the MLH1/3 foci are consistently associated with RNs at the sites of chiasmata, we circumvent the chronological disparity between RN-MLH1/3 presence and chiasma detection by inducing a precocious chiasmate diplotene stage with the phosphatase inhibitor, okadaic acid (OA; Wiltshire et al. 1995; Tarsounas et al. 1999). In the presence of okadaic
acid, the SYN1 protein that holds the lateral elements in parallel alignment is degraded so that the lateral elements initiate precocious separation except at the sites where the homologous chromosomes are held together by the reciprocal exchange events, which is apparent by the inflection or convergence of the chromosome cores. The inflection of the chromosomes at the site of a crossover is apparent in Figure 1 and the corresponding immunofluorescence image of chromosome cores and 4',6-diamidino-2-phenylindole (DAPI)-stained chromatin is shown in Figure 3 (Moens and Spyropoulos 1995). With fluorescent and electron microscope observations, we now confirm that the MLH1/3 proteins are localized to the RNs of OA-treated spermatocytes and that the RN-MLH1/3 structures are localized to the precociously induced chiasmate structures.

**Figure 1.**—Mouse oocyte chromosomes differentially labeled by BrdU incorporation at the penultimate S-phase prior to meiosis demonstrate that a chiasma is the site of a crossover and that the inflection of the chromosomes corresponds to the site of a chiasma/crossover (with permission from Polani 1981 and Traut 1991).

**Figure 2.**—An MLH1 focus in the synapsed segment of a diplotene bivalent suggests but does not confirm the position of a crossover (taken from Figure 6A, arrow).

**Figure 3.**—Immunofluorescent cytology of chiasmata in untreated mouse diplotene spermatocytes. The chromosome core proteins are visualized with COR1 antibody, the synaptic protein with anti-SYN1 serum, the centromeres with CREST serum, and the chromatin with DAPI. (A) A typical diplotene bivalent with bright centromeres surrounded by centromeric heterochromatin, FITC-labeled cores, and patches of residual SYN1 protein along the cores. The chromatin lacks the condensation that is induced in traditionally fixed chromosomes (Figure 1) and chromatin domains extend between the chromosome cores including the site of inflection, the presumptive crossover/chiasma. (B and C) The formation of a chiasma. (B) The parallel aligned cores (orange lines) are interconnected by SYN1 protein (vertical black lines). (C) When the SYN1 protein is degraded, the cores separate except at the site of a chiasma/crossover, which can also be the site of an RN and MLH1/3 protein (arrow). (D) Frequently, the SYN1 protein is present at the site of last contact between the cores and at the points of inflection. The centromeres are also stained with rhodamine but can be differentiated from SYN1 sites by the blue centromeric heterochromatin.

**Figure 4.**—Conventional appearance of SC-associated MLH1/3 immunofluorescent foci. (A and B) Untreated and treated pachytene spermatocytes, respectively. MLH1p, green; COR1p and histone H1t, red. (C and D) Untreated and treated pachytene spermatocytes, respectively. MLH3p, green; COR1p, red. The SCs are 10–20 μm in length.
Figure 5.—Electron micrographs of SCs, RNs, and MLH1p with explanatory diagrams below. (A) Okadaic-acid-treated spermatocytes at 0 hr. The SC has the normal appearance of two parallel aligned lateral elements. The RN has a 5-nm immunogold anti-MLH1p label (enlarged in the inset). (B) At 2 hr OA treatment, SCs have separated lateral elements, which are still held together at the sites of the RN-MLH1 structure (arrow). The 15-nm gold grains are background from an anti-centromere protein antibody. (C) At 4 hr OA treatment, the chromosome cores are widely separated and the RN-MLH1 structure is located between the cores where they converge. The width of the normal SC is ~200 nm.

Purified zygotene/early pachytene spermatocytes were prepared according to Heyting et al. (1985). Mouse spermatocytes were isolated from 30-day-old mice by centrifugal elutriation and density gradient. Cells were plated in minimal essential medium supplemented with 2–5 μl OA (Sigma, St. Louis) from 1 mm stock in ethanol at a density of 5 x 10^6/ml and cultured at 32°C with 5% CO2. At 1–4 hr, cell samples were withdrawn (Tarsounas et al. 1999). For microspreading, 1-μl drops were touched to each of the drops of 40-μl hypotonic salt (0.5%) solutions on the 12 wells of albumin-coated (2%) multiwell slides. After settling for 20 min, the nuclei were fixed in 2% paraformaldehyde with 0.03% SDS for 3 min and washed for 1 min three times in 0.04% Photo-Flo 200 (Kodak) and left to dry a few minutes. To recognize the chromosome cores, rabbit anti-COR1 serum from rabbit D was used (Dobson et al. 1994). The MLH1 foci were detected with commercial mouse anti-MLH1 protein (BD-Bioscience). The MLH3p antibody has been characterized by Lipkin et al. (2002) and was provided by P. Cohen. Antibody to testis-specific histone H1t was used to detect the progression from zygotene into the pachytene stage where H1t is expressed. After blocking, the primary antibody was applied overnight and, after washes, the secondary FITC or rhodamine-conjugated secondary antibodies were applied for 1 hr at 37°C. For electron microscopy, the nuclei were attached to plastic film and immunostained with immunogold. The film was floated off on water, picked up on EM grids, and viewed/recorded at 10 kV on the negatives.

The isolation procedures and the OA treatment at 0 and 1 hr do not affect the detection of immunofluorescent MLH1 foci (Figure 4, A and B) and MLH3 foci (Figure 4, C and D). These two types of foci have been shown to colocalize (Lipkin et al. 2002). The localization patterns of MLH1p and MLH3p on the OA-treated chromosome cores did not differ from the untreated spermatocytes. Typically some 23–26 MLH1/3 fluorescent foci are associated with the pachytene SCs in a nonrandom distribution of 1 or 2 foci per SC (Figure 4; Anderson et al. 1999; Hassold et al. 2000; Koehler et al. 2002).

RN-MLH1 structures can be identified in electron micrographs of control and okadaic-acid-treated spermatocytes (Figure 5, A–C). At 0 hr OA treatment, the SCs have the normal appearance of two parallel aligned lateral elements and they contain one or two electron-dense RNs, which are recognized by the anti-MLH1p antibody (Figure 5A and inset). At 2 hr OA treatment, the chromosome cores have initiated separation and the RN-MLH1 structure is at the site where the cores are still held together (Figure 5B and inset). At 4 hr OA treatment, the cores are fully separated and the RN-MLH1 structure is present between the cores where they converge (Figure 5C and inset). These results show that MLH1p is present at the RNs and chiasmata and there-
fore support the idea that immunofluorescent MLH1 foci may be used as the markers for crossovers and chiasmata.

The precocious onset of OA-induced chromosome core separation at the time that MLH1/3 foci are still fully observable provides the opportunity to immunocyto- logically demonstrate the localization of RN-MLH1/3 structures to the sites of mature chiasmata of a given spermatocyte nucleus (Figure 6). In classical chiasmate bivalents, the interstitial chiasmata are characterized by inflections of the separated chromosomes/cores and terminal chiasmata by convergence of the terminal segments of the chromosomes/cores (Figures 1 and 3; Moens and Spyropoulos 1995). In untreated nuclei, the inflection of cores might be attributed to the presence of the synaptic protein SYN1 at those sites (Figure 3D) rather than to crossover events. However, in OA-treated spermatocytes, the SYN1 protein is rapidly degraded (Tarsounas et al. 1999) and a crossover is therefore the more likely cause of cohesion between homologous cores. Furthermore, when the distance between the inflections exceeds the 200-nm span of the SYN1 protein (Figure 6, C and H), a crossover rather than the SYN1 protein is the more likely cause of the inflection.

During the progression from early diplotene to metaphase I, the numbers of MLH1/3 foci are reduced from ~26 to 0. The percentage of okadaic-acid-induced diplo-
tene nuclei with high, medium, and numbers of foci examined is shown in Figure 7. For MLH1p, 165 nuclei were analyzed and, for MLH3p, 84 nuclei. Most of the MLH1 foci are present in association with the precociously induced chiasmate state of the two nuclei at 2 hr of OA treatment (Figure 6A). At a higher magnification, it is evident that the MLH1 foci are present between the inflections of the cores in Figure 6, B and C, for MLH1p and in Figure 6, G and H, for MLH3p. MLH1 foci are at the points of last contact in Figure 6, D–F. These observations constitute the formal proof that immunofluorescent MLH1/3 foci mark the sites of crossover/chiasmata.

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LITERATURE CITED


