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The association of ATR protein with mouse meiotic chromosome cores

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Abstract. The ATR (ataxia telangiectasia- and RAD3-related) protein is present on meiotic prophase chromosome cores and paired cores (synaptonemal complexes, SCs). Its striking characteristic is that the protein forms dense aggregates on the cores and SCs of the last chromosomes to pair at the zygotene-pachytene transition. It would appear that the ATR protein either signals delays in pairing or it is directly involved in the completion of the pairing phase. *Atm*-deficient spermatocytes, which are defective in the chromosome pairing phase, accumulate large amounts of ATR. The behaviour of ATR at meiotic prophase sets it apart from the distribution of the RAD51/DMC1 recombinase complex and our electron microscope observations confirm that they do not co-localize. We failed to detect ATM in association with cores/SCs and we have reported elsewhere that RAD1 protein does not co-localize with DMC1 foci. The expectation that putative DNA-damage checkpoint proteins, ATR, ATM and RAD1, are associated with RAD51/DMC1 recombination sites where DNA breaks are expected to be present, is therefore not supported by our observations.

Introduction

The ATM (ataxia telangiectasia-mutated), ATR (AT- and RAD3-related) and mRAD1 (*Schizosaccharomyces pombe* RAD1 homologue) proteins have been implicated in mammalian checkpoint processes that can cause a delay in the somatic cell cycle and cell death following DNA damage (Keegan et al. 1996; Freire et al. 1998; Wright et al. 1998). These proteins have been reported to occur at the chromosome cores and synaptonemal complexes (SCs) of meiotic prophase chromosomes but their functions in the meiotic pathway are not well defined (Keegan et al. 1996; Flaggs et al. 1997; Freire et al. 1998).

Because the high levels of meiotic recombination require numerous single and/or double-strand DNA breaks, the possibility exists that checkpoint proteins are closely associated with the sites of recombination processes to monitor proper maturation of the joined molecules before meiotic development continues (Xu et al. 1997). To test this hypothesis at the structural level, we have examined immunocytologically the extent to which the recombination protein complex RAD51/DMC1, which is localized at the chromosome cores/SCs (Bishop et al. 1992; Shinohara et al. 1992; Ogawa et al. 1993; Ashley et al. 1995; Haaf et al. 1995; Terasawa et al. 1995; Anderson et al. 1997; Moens et al. 1997), co-localizes with putative checkpoint proteins ATM, ATR, and RAD1. For RAD1 foci, we found no persuasive evidence for such co-localization (Freire et al. 1998). We do not detect the ATM protein in spermatocyte spreads (Barlow et al. 1998). Here we report that the ATR protein also has no convincing association with the RAD51/DMC1 complex.

We conclude that the putative DNA-damage checkpoint proteins, ATR, ATM and RAD1, do not necessarily occur at the RAD51/DMC1 recombinase foci where the DNA breaks are expected to be present. Instead, our immunocytological data suggest that ATR may play a role in the completion of chromosome pairing at the zygotene-pachytene transition.

Materials and methods

Antibodies. The coding region of mouse *DMC1* cDNA was cloned in the pET3a vector (Habu et al. 1996) and the full-length DMC1 protein with an N-terminal $(His)_6$ tag was expressed in *Escherichia coli*. The recombinant protein was purified on Ni-agarose (Qiagen, USA) and injected interperitoneally into mice. The polyclonal antibody was depleted of RAD51 cross-reacting antibodies by adsorption to Sepharose-immobilized RAD51 protein. The RAD51 protein was overexpressed in *E. coli* as described (Habu et al. 1996) and affinity purified on a Ni matrix. The antibodies to meiotic chromo-

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some cores/SCs and to centromere proteins have been reported previously (Dobson et al. 1994) and the anti-RAD51 antibody and its localization to cores/SCs were described in Moens et al. (1997).

Three regions of the ATR spanning amino acid residues 2122–2380, 2381–2644 and 2122–2644 were amplified from reverse-transcribed HeLa cell mRNA by standard procedures. Clones were inserted in-frame with the hexa-histidine tag of the vector pQe-30 (Qiagen, USA) and were expressed and purified according to the manufacturer's instructions. Antibodies against the recombinant proteins were raised in rabbits by standard procedures (Harlow and Lane 1988). Depletion of anti-ATR antibody with the corresponding antigen abolished all ATR immune staining on the cores, SCs and sex vesicles of mouse spermatocytes.

Immunocytology. Immunofluorescence and immunogold methods were the same as reported for RAD51 immunocytology (Moens et al. 1997). Briefly, spermatocytes were attached to plastic-coated glass slides (dipped in 0.5% Falcon plastic in chloroform) and fixed in 2% paraformaldehyde. After washes that contain blocking serum in PBS, cells were incubated for 2 h at 37° C in primary antibody at empirically determined dilutions to maximize signal and minimize background. Washes were performed as before and secondary antibody incubation was for 1 h at 37° C [fluorescein isothiocyanate (FITC), rhodamine, 5, 10 and 15 nm gold particles]. Following washes in PBS and a final wash in water with Kodak Photo-Flo, slides were dried and mounted with ProLong antifade (Molecular Probes, Eugene, Pre.) for epifluorescent microscopy or the plastic film was floated off, loaded with electron microscope grids and dried. Images were recorded on photographic film.

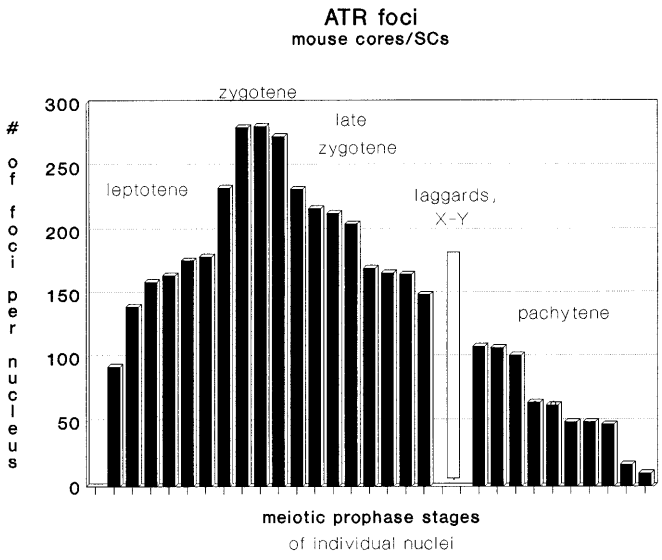


Fig. 1. ATR foci during meiotic prophase development in the male mouse. The foci appear coincient and associated with chromosome core formation. The number rises to about 280 at the time that synapsis of the cores is in progress. The number then drops but at the end of the pairing/synaptic phase, a heavy concentration of ATR antigen appears on the cores and synaptonemal complexes (SCs) of autosomes that are the last to pair (laggards) and on the X–Y chromosomes. When synapsis is complete, the number declines to zero on the autosomal SCs while the X–Y chromosomes and the sex vesicle become heavily coated with ATR antigen (see Fig. 2)

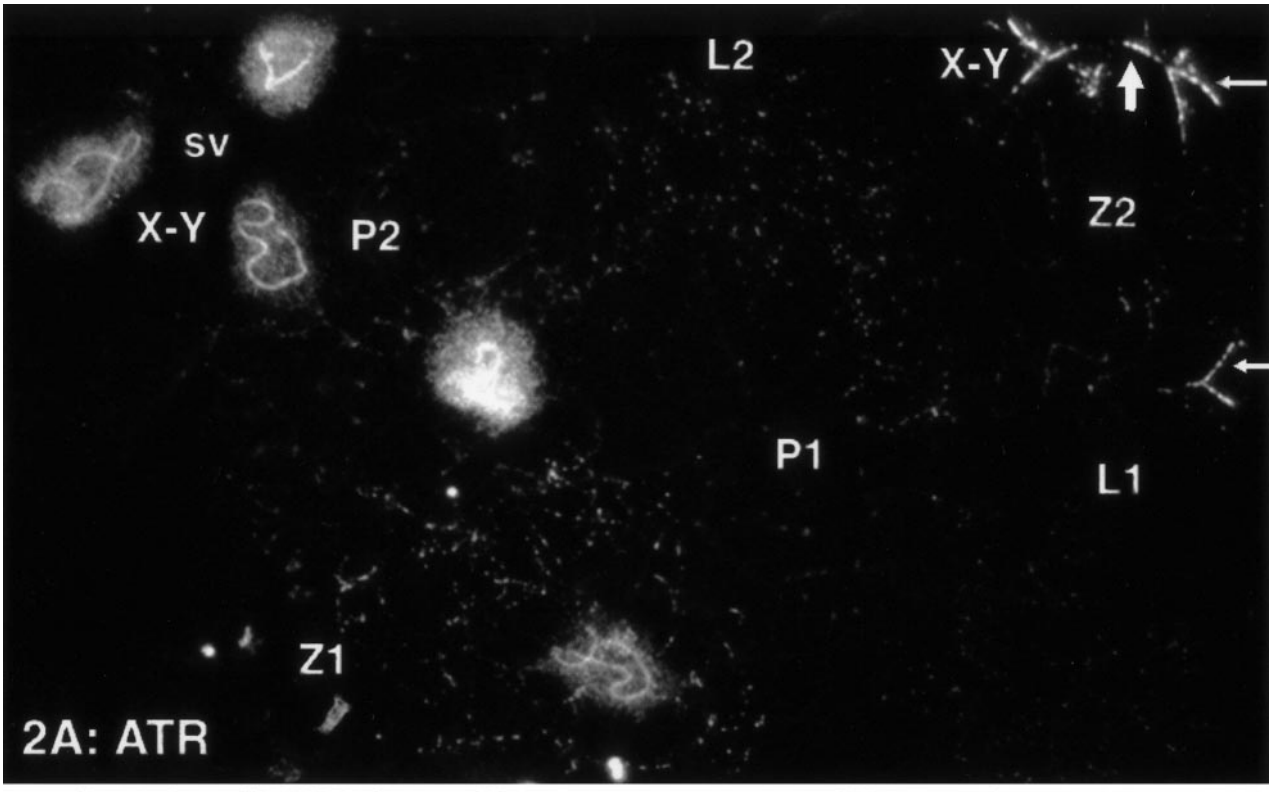
Results

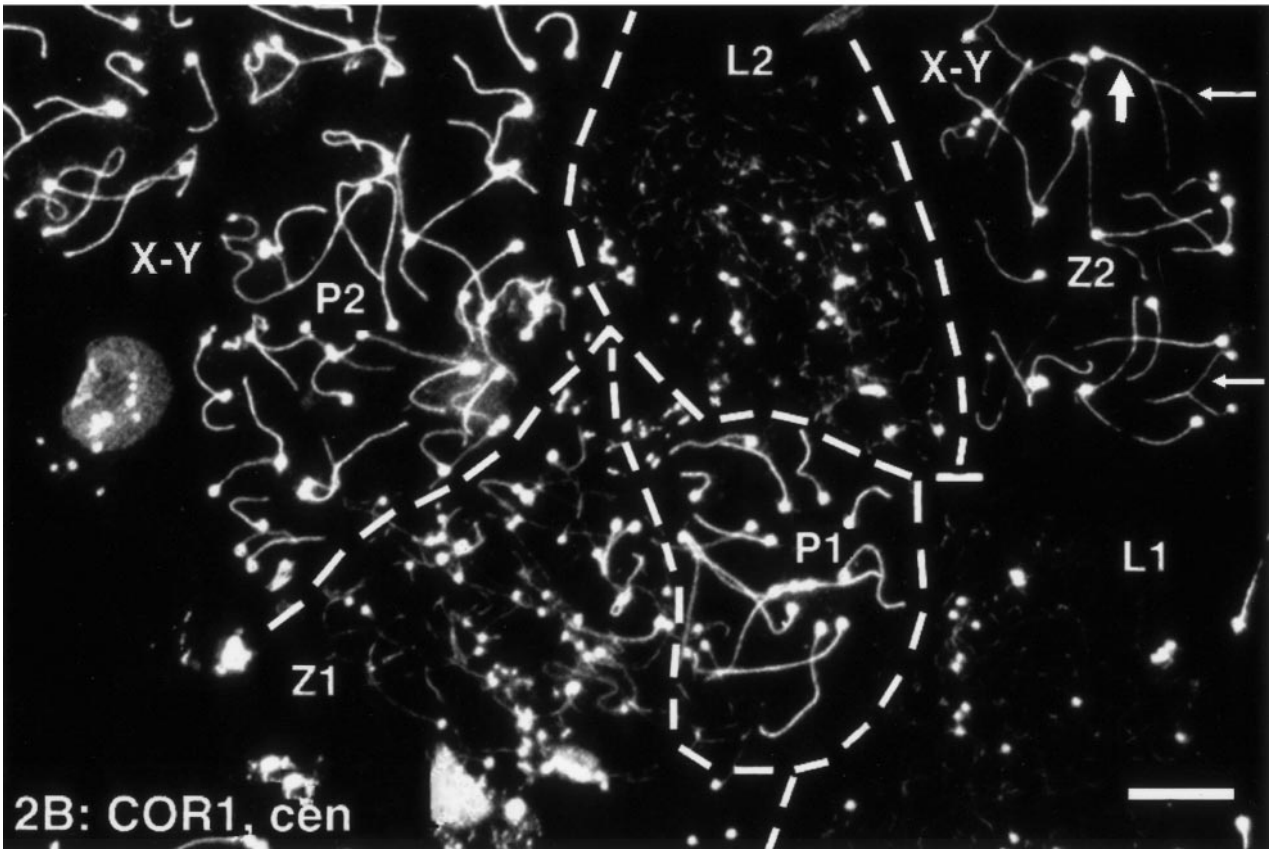
Distribution of ATR at prophase I of meiosis in wild-type and Atm-deficient mice

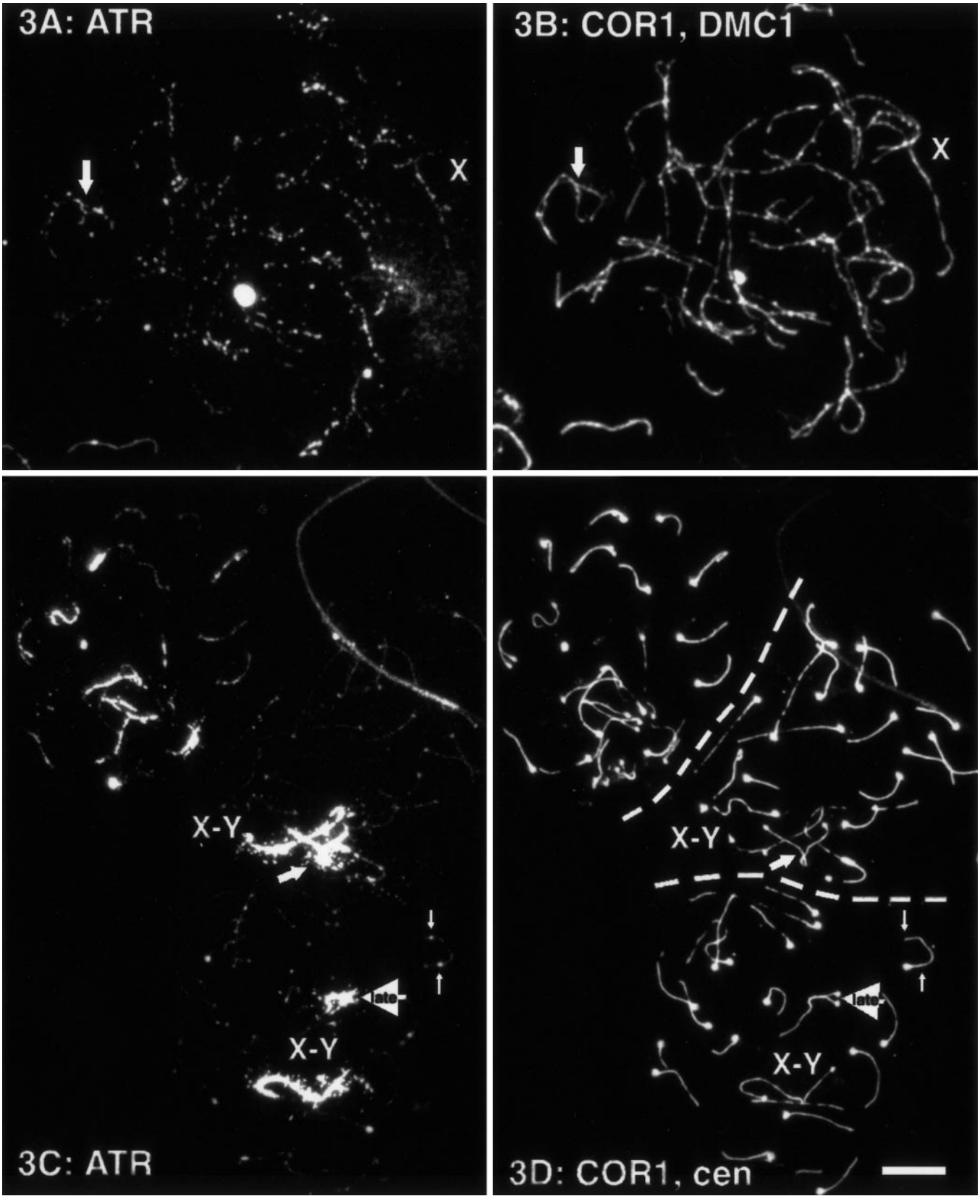
In order to compare the reported location and time course of appearance of RAD51/DMC1 foci (Moens et al. 1997) with the dynamics of ATR foci, the number and position of ATR foci were recorded at successive stages of meiotic prophase. The ATR antigen is first detected in association with newly formed chromosome cores of leptotene nuclei. The number of immunofluorescent foci increases to approximately 280 at the beginning of the synaptic stage, zygotene. The number then declines as zygotene progresses (Fig. 1) but there is a sharp rise in the concentration of foci on the last few chromosomes that have not completed pairing, as well as on the unpaired X–Y chromosomes. The staging of meiotic prophase nuclei is based on morphology of chromosome cores, SCs and number of centromeres (40 singles prior to zygotene, reduced to 19 doubles plus X–Y singles at pachytene).

The abundance and distribution of ATR foci in spermatocyte nuclei at different meiotic prophase stages are demonstrated in Fig. 2. There are six nuclei in Fig. 2A that are immunostained with anti-ATR antibody and FITC-conjugated secondary antibody. The cores/SCs/centromeres of the same nuclei are visualized with rhodamine fluorescence in Fig. 2B. The early leptotene nucleus (Fig. 2B, nucleus L1) has short core segments and unpaired centromeres and it has about 140 ATR foci (Fig. 2A, nucleus L1). In the somewhat more advanced leptotene nucleus, L2, there are more core segments but the centromeres are still mostly single. The ATR foci increase to about 165 foci and they are brighter than in L1. Synapsis is in progress in nucleus Z1 where there are single and paired centromeres and the approximately 170 ATR foci are evenly distributed over cores and SCs. The X–Y pair is not yet differentially labelled. (The bright X–Y pair in the Z1 nucleus belongs to pachytene nucleus P1, which overlaps partially with Z1 as a result of dispersion during the surface-spreading procedure). The chromosomes of the late zygotene nucleus, Z2, are mostly synapsed except for the two bivalents marked by

Fig. 2A, B. Six mouse spermatocyte nuclei, *L1, L2, Z1, Z2, P1* and *P2*, immunostained for ATR antigen and visualized with fluorescein isothiocyanate in **A** and with rhodamine for chromosome cores, SCs and centromeres in **B**. The nuclei are in successive stages of meiosis, leptotene (L1, L2), zygotene (Z1, Z2) and pachytene (P1, P2) and they demonstrate the abundance and distribution of ATR antigen at the meiotic prophase stages. L1 is part of an early leptotene nucleus with short core segments, unpaired centromeres and about 140 ATR foci. Nucleus L2 is a slightly more advanced leptotene nucleus with single centromeres and about 165 brighter foci. Z1 is an early zygotene nucleus with paired and unpaired centromeres and with about 170 foci. Foci are evenly distributed over synapsed and presynaptic cores (the bright X–Y chromosomes belong to the nearby P1 nucleus). Z2 is essentially a pachytene nucleus except for two chromosomes, that are only partially paired (*small arrows*). The unpaired as well as the paired segments (*large arrow*) are heavily labelled. The massive loading of ATR protein on the laggards and the X–Y chromosomes is the structurally most dominant characteristic of ATR antigen distribution at meiotic prophase. Completely synapsed cores have a few ATR foci. Pachytene nuclei, P1 and P2, have heavily labelled X–Y chromosomes and sex vesicles (*SV*). There is no evidence that the pseudo-autosomal region is specifically labelled at any time, thereby rendering a direct involvement of ATR protein in recombination uncertain. *Bar* represents 10 μm


L2
X-Y
sv
X-Y
P2
Z2
P1
L1
Z1
2A: ATR


L2
X-Y
X-Y
P2
Z2
P1
L1
Z1
2B: COR1, cen


3A: ATR
X
3B: COR1, DMC1
X
X-Y
late
X-Y
3C: ATR
X-Y
late
X-Y
3D: COR1, cen

thin arrows. The cores and SCs (thick arrow) of these late-pairing laggards and the cores of the X–Y pair are strongly labelled by the anti-ATR antibody. Observations of equivalent meiotic nuclei of female mouse demonstrate no exceptional labelling of the synapsed X–X sex chromosomes (not shown). The pachytene nuclei, P1 and P2, of Fig. 2 have few, if any, autosomal foci but the X–Y pair and the sex vesicle are strongly fluorescent.

The distinctive distribution of ATR protein during the synaptic phase is detailed in the zygotene nuclei of Fig. 3. During early and mid-zygotene, the ATR foci (Fig. 3A) are evenly distributed along single and synapsed cores (Fig. 3B). The pairing forks (arrow) and the X chromosome, X, are not differentially labelled at the early/mid-zygotene stages. Three nuclei at late zygotene (Fig. 3C, D) demonstrate that the last cores to pair and the X–Y chromosomes are heavily coated with ATR antigen (large arrow, late). These late-pairing cores are fully formed and denser than the early zygotene cores and to make that distinction we refer to them as laggards. Because the cores are far apart rather than in close proximity, we prefer to call this event late 'pairing' rather than late 'synapsis' since synapsis is still to follow.

The fact that late pairing rather than normal pairing is responsible for the over-abundance of ATR protein is demonstrated by the lack of ATR aggregates during normal pairing at zygotene as shown in Fig. 4A, B. In the four *Atm*-deficient nuclei with pairing defects (Fig. 4A B), there are numerous partially synapsed cores that fail to complete synapsis (Barlow et al. 1998). These numerous laggards may account for the heavy ATR labelling of the nuclei. Furthermore, the ATR protein may also be associated with non-homologously synapsed cores, which are common in these nuclei.

ATR versus DMC1 foci

The physical association of ATR foci with DMC1 foci was examined with two-colour immunofluorescence (Fig. 3A, B) and electron microscopy (Fig. 5). In Fig. 3A, the ATR foci of a zygotene spermatocyte nucleus are visualized with FITC fluorescence and the cores/SCs/DMC1 foci of the same nucleus are seen with rhodamine fluorescence in Fig. 3B. Comparison of Fig. 3A with 3B demonstrates that at this high density of foci and at this level of resolution, some of the 280 ATR foci appear to co-locate with the 290 DMC1 foci. This co-location is fortuitous because, at the resolution of electron microscopy (Fig. 5), it is apparent that, while close together, the two types of foci, ATR and DMC1, are mostly separate. The relative locations of ATR and DMC1 antigens along the meiotic chromosome cores/SCs are evident from electron micrographs in which the two antigens are differentially labelled with 5- and 10-nm gold particles (Fig. 5A, B). Figure 5A is an example of a late-zygotene nucleus with a generally low level of ATR labelling of the SCs but heavy labelling at the late-pairing fork, which is the distinguishing characteristic of ATR localization. In addition, it is evident from Fig. 5B that the ATR antigen also occurs in association with the synapsed portion of the cores. The 5-nm grains tagging the DMC1 antigen are not intermingled with the 10-nm grains of the ATR antigen, indicating that the two proteins are not usually co-localized. However, the close proximity of the two types of antigen evident in the electron micrographs of Fig. 5 would, in the case of fluorescent immunocytology, give the misleading impression of co-localization. Figure 5B gives clear evidence that, contrary to Keegan et al. (1996), ATR antigen is still present alog fully synapsed chromosome cores.

Fig. 3A–D. Immunofluorescent comparison of ATR foci at mid-zygotene (**A, B**) with the distribution of foci at late zygotene (**C, D**). **A** Distribution of some 290 ATR foci in a mid-zygotene mouse spermatocyte. Cores and synapsed cores (*arrow*) have a similar abundance of foci and there are no particularly heavily labelled autosomal or sex chromosome (*X*) cores(SCs). **B** The same cores/SCs are immunostained with anti-COR1 and anti-DMC1 to demonstrate the progression of chromosome synapsis and that some of the 270 DMC1 foci appear to coincide with the ATR foci. However, this is not evident with electron microscopy (see Fig. 5). **C, D** Three surface-spread nuclei at the end of the synaptic process show evidence that cores /SCs of late-pairing bivalents are heavily coated with ATR antigen (*large arrows, late*). The late-pairing chromosomes are referred to as laggards because the heavily ATR-labelled cores (*broad arrow*) are not typical of cores normally seen at zygotene (Fig. 3B). Fully snapsed bivalents have few but distinct ATR foci at the SCs (*small arrows*). The X–Y cores and surrounding chromatin have similar amounts of ATR to the laggards. *Bar* represents 10 μm

Discussion

In comparison with the distribution of RAD51/DMC1 the ATR antigen has the exceptional characteristic at meiotic prophase that it accumulates heavily on the late-pairing chromosome cores at the zygotene-pachytene transition (Figs. 2A, 3C). At early and mid-zygotene stages, the foci are evenly distributed along cores and SCs and no excessive accumulation is evident in conjunction with the pairing process (Fig. 3A, B). Apparently it is the incomplete pairing at late-zygotene stages that induces the accumulation of ATR antigen on autosomal laggards as well as on the X and Y chromosomes. In normal meiosis, there are usually only a few laggards (Figs. 2, 3) but in the *Atm*-deficient mutant with pairing defects, there are numerous partially paired and mispaired chromosomes and these are associated with copious amounts of ATR antigen (Fig. 4) (Barlow et al. 1997, 1998). Thus, the distribution and time course of appearance of ATR foci differs significantly from those of RAD51/DMC1 foci by the fact that the two types of foci do not normally co-locate (Figs. 3A, 5) and by the fact that ATR foci are highly concentrated on late-pairing chromosomes. The differences suggest that these proteins may also perform functions that are not directly related. RAD51/DMC1 are combined in this discussion because the two proteins are detected in the same foci with electron microscopy of differential immunogold labelling (Tarsounas et al., submitted).

Keegan et al. (1996) report that ATR and ATM "… show complementary localization along synapsing chro-

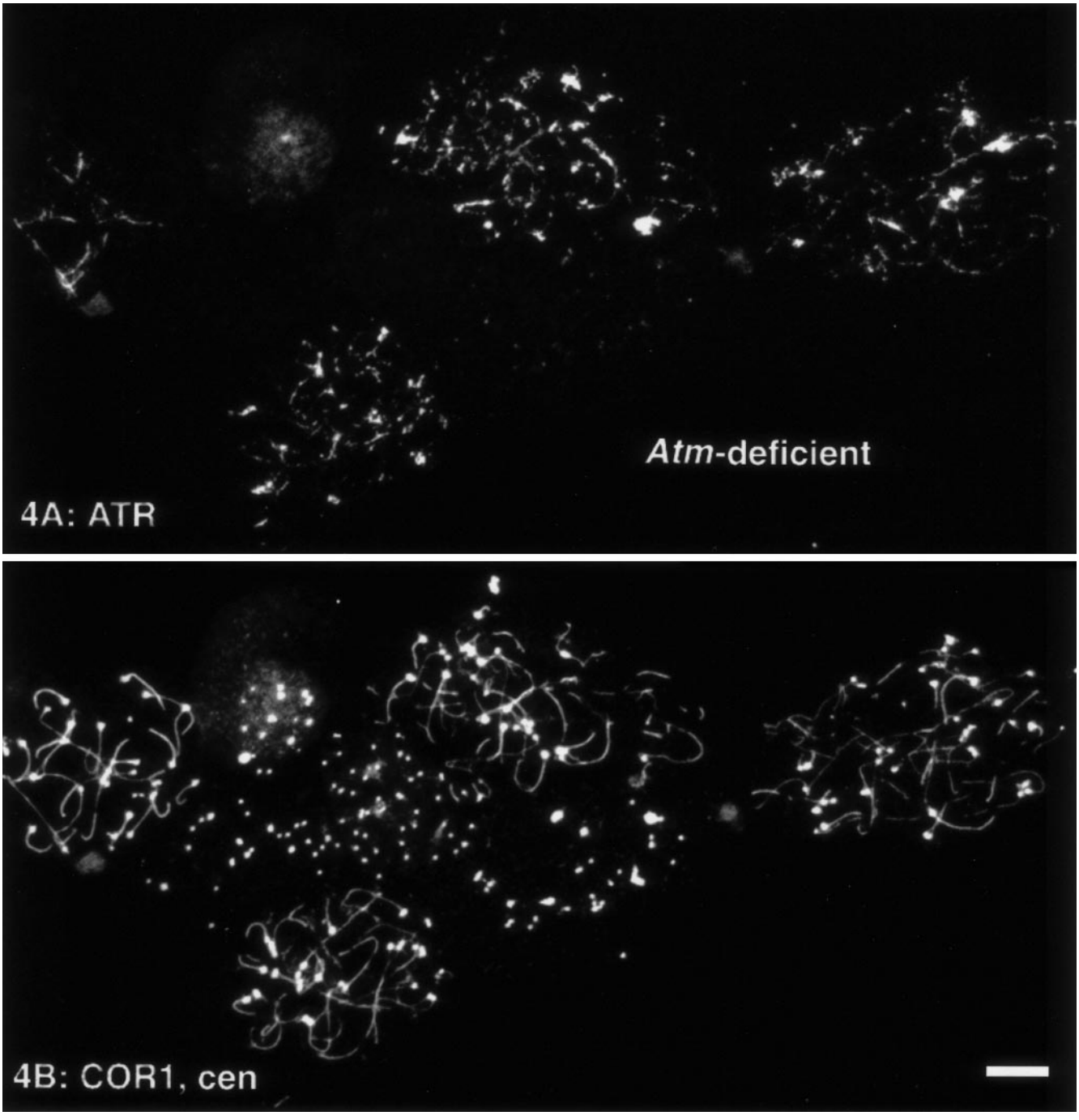


Fig. 4A, B. Spermatocytes of *Atm*-deficient mice are reported to have pairing defects. There are numerous incompletely paired and mispaired chromosomes and coincidently there is an excessive amount of ATR antigen observed in such nuclei. **A** ATR foci in *Atm*-deficient meiotic prophase nuclei. Instead of just one or two laggards being labelled, many of the cores and SCs are heavily coated with ATR antigen. **B** The same nuclei with rhodamine-stained cores/SCs and centromeres. As a result of extensive mispairing, these nuclei do not produce well-defined pachytene SCs. *Bar* represents 10 μm

mosomes. Atr is found at sites along unpaired or asynapsed chromosomal axes whereas Atm is found along synapsed chromosomal axes." Our observation on ATR foci indicates that it is not exclusively associated with unpaired cores. Instead, we observe the antigen on cores and SCs at early and mid-zygotene. At late zygotene, the cores and some of the SCs of late-pairing chromosomes are heavily labelled. At pachytene, SCs have few, but distinct, ATR foci. We have tested five anti-ATM antibodies that recognize ATM on immunoblots but we cannot detect foci in association with chromosome cores or SCs of spermatocytes and we find no differences in the staining patterns of normal and *Atm*-deficient spermatocytes (Barlow et al. in press). We conclude that, in the absence of ATM foci, no inference can be made about the complementarity or exclusivity of ATR and ATM foci at meiotic prophase.

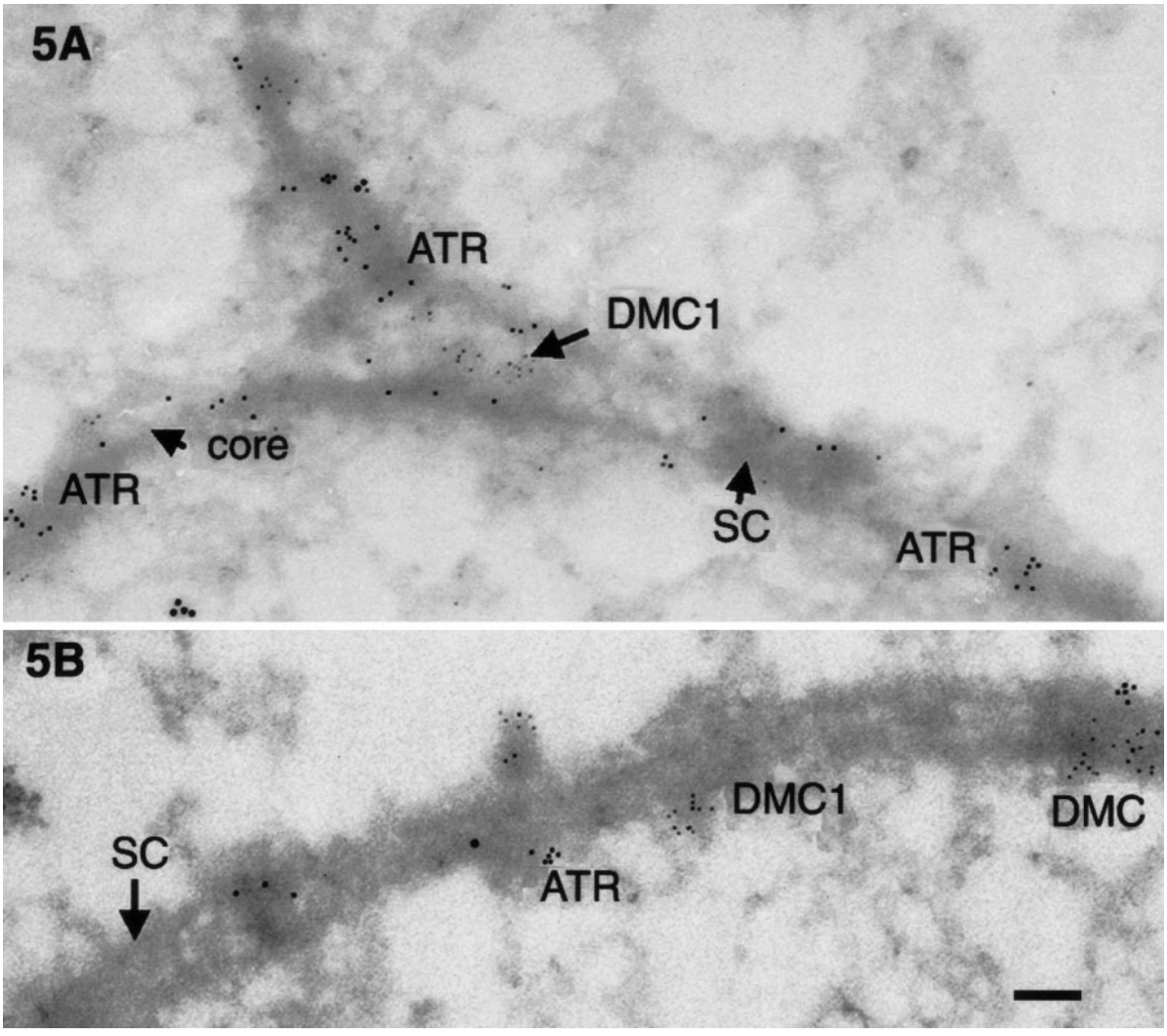


Fig. 5A, B. Electron micrographs of chromosome cores/SCs with 10-nm gold grains marking ATR antigen and 5-nm grains at DMC1 sites. The two types of antigens are mostly separate from each other. **A** A late pairing fork with extensive 10-nm anti-ATR labelling at the fork and a few aggregates of 5-nm grain labelled DMC1. **B** A pachytene SC with ATR and DMC1 aggregates. Some of these would be perceived to be co-localized by fluorescent microscopy even thought the sites are quite distinct at the resolution of electron microscopy. *Bar* represents 200 nm

DMC1 is a RECA-type protein that functions in recombination (Bishop et al. 1992; Bishop 1994) and specifically monitors and enforces an interhomologue bias (Schwacha and Kleckner 1997). In that capacity, it also appears to play a dominant role in chromosome synapsis. The *Dmc1*-deficient mutant mouse fails to synapse homologues at meiotic prophase and the synaptic protein, recognized by the anti-SYN1 antibody, is not assembled between homologous cores (Pittman et al. 1998; Yoshida et al. 1998). Thus, even though DMC1 and ATR are both associated with pairing and synapsis, the differential timing and localities of the two proteins at the meiotic prophase chromosome cores/SCs suggest that the two proteins play different roles in pairing and synapsis.

In *Saccharomyces cerevisiae*, genetic and cytogenetic evidence suggests that meiotic cells monitor the status of SC formation (Tung and Roeder 1998). ATR could play such a role in the surveillance of synaptic completion in mouse. This possibility is supported to some extent by the observation that in *Atm*-deficient mouse spermatocytes which arrest at zygotene (Barlow et al. 1997; Xu et al. 1997), excessive amounts of ATR accumulate in the affected nuclei. This interpretation is at variance with our expectation that DNA breaks resulting from the recombination process, and which are presumably marked by the RAD51/DMC1 recombinases, would be associated with putative DNA-damage detection and signalling proteins. In this report, we show that the ATR protein does not co-locate with the RAD51/DMC1 complex and we have previously shown that RAD1 foci do not co-locate with RAD51/DMC1 foci (Freire et al. 1998). Furthermore, we note that ATM, also a potential signal of DNA damage, is not detectable immunocytologically at the sites of RAD51/DMC1 protein. The general conclusion is that there is no simple relationship between sites of recombination and the DNA-damage detection/signalling machinery.

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