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The meiosis-specific *Xmr* gene product is homologous to the lymphocyte Xlr protein and is a component of the XY body

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The *Xlr* (X-chromosome linked, lymphocyte regulated) multigene family was previously found to determine, in the lymphoid cell lineage, the stage-specific expression of a nuclear protein with a primary sequence suggestive of a transcriptional activator function. We report here the characterization of a second functional member of the *Xlr* gene family that is abundantly transcribed in testis in a tissue-specific and developmentally regulated manner. The protein product of this newly identified gene, called *Xmr* (Xlr-related, meiosis regulated), is located in the nuclei of spermatocytes, early in the prophase of the first meiotic division, and later becomes concentrated in the XY nuclear subregion where it is in particular associated with the axes of sex chromosomes. The Xmr protein provides a new tool for the investigation of sex chromosome behaviour during meiosis in mammals.

Key words: meiosis/nuclear protein/XY pair/X-chromosome/Xlr gene family

Introduction

Sexual reproduction in eukaryotic organisms involves a specific form of cell division known as meiosis. During this process, correct pairing of homologous chromosomes allows DNA recombination and reduction of a diploid number of chromosomes to a haploid one, a new diploid genome being constituted after fertilization (reviewed in von Wettstein *et al.*, 1984). Meiotic chromosomes show an evolutionarily conserved behaviour including condensation followed by synapsis between homologues and eventually ordered segregation, all of these occurring along a precise time pattern of cell differentiation. In all species studied to date, these changes affect all pairs of autosomes in a very similar manner. In meiosis in male mammals, however, the sex chromosomes behave differently (reviewed in Solari, 1974). Their pairing is delayed relative to that of autosomes and it affects only part of their length while they are inactivated. Remarkably, these events occur in a specific area of the nucleus which is easily discerned morphologically and is termed the XY body or sex vesicle. The formation of the XY body has been attributed to the heteropycnotic behaviour of sex chromosomes whose condensation begins at zygotene, increases until mid-pachytene and remains unchanged until diplotene whereas autosomal chromatin undergoes a complex cycle of condensation as early as leptotene followed by decondensation at mid-pachytene and recondensation at diplotene.

Although the XY body was identified almost a century ago (Lenhosšék, 1898), its detailed description has relied almost exclusively on light and electron microscopy observation and no molecular component was ascribed to it until very recently (Smith and Benavente, 1992b). Neither is its function known, although it can be assumed to be important. This nuclear substructure is conserved across the mammalian phylogenetic tree and, in eutherian mammals, is intimately associated with ongoing XY pairing and X inactivation. Convincing arguments indicate that these latter two processes play a critical role in spermatogenesis in *Drosophila* (Lifshytz and Lindsley, 1972; Coyne, 1985; McKee and Karpen, 1990) as well as in mammals (Burgoyne *et al.*, 1992a,b). Factors that interfere with XY pairing all result in spermatogenic failure (Solari, 1971; Burgoyne *et al.*, 1986; Tease and Cattanach, 1989; Matsuda *et al.*, 1991; Sutcliffe *et al.*, 1991). In all these cases, the spermatogenic block occurs primarily at the pachytene stage of prophase-1 or in metaphase-1. Since it circumscribes a particular domain of the nucleus where key XY-associated events take place, the XY body most likely plays an essential role in spermatogenesis.

The murine *Xlr* (X-linked, lymphocyte regulated) multigene family was originally identified by means of subtractive DNA hybridization and cloning (Cohen *et al.*, 1985a,b). A predominant transcript called pM1 was characterized in lymphoid cell lines and found to be expressed in a developmentally regulated manner (Cohen *et al.*, 1985b; Siegel *et al.*, 1987). The amino acid sequence deduced from the nucleotide sequence predicted the existence of a 25 kDa protein, which showed no close sequence similarity with any known protein. It contained, however, an N-terminal acidic stretch of 45 residues suggestive of a transcriptional activator function (Ma and Ptashne, 1987; Gill and Ptashne, 1987) while the primary sequence of the C-terminal half was compatible with a coiled-coil structure. By using antibodies raised against the bacterially expressed protein, the product of the Xlr open reading frame (ORF) was found to be located in the cell nucleus (Garchon and Davis, 1989). These characteristics suggested that the Xlr protein could be a nucleic acid-binding protein and could be involved in gene regulation.

We now report the cloning and the sequencing of the cDNA of a second functional member of the *Xlr* family. This new gene, which we call *Xmr* (Xlr-related, meiosis regulated), is abundantly expressed in a strictly regulated manner in the nuclei of mouse primary spermatocytes. During prophase-1, its protein product progressively becomes concentrated in the XY nuclear subregion.

Results

Abundant transcription of an Xlr-related gene in adult mouse testes

A panel of RNA samples from various organs of adult (8 week old) mice was screened by Northern blot hybridization using the pM1 full-length *Xlr* cDNA probe. As shown in Figure 1A, an *Xlr*-related message could easily be visualized in testis RNA, whereas no signal was detected in any of the other tissue samples, even after prolonged exposure of the filter. The pM1 transcript was shown to belong to the class of rare mRNAs; a frequency of 5–15 *Xlr* mRNA molecules per lymphoid cell was previously estimated (Siegel *et al.*, 1987). By comparison with V17C, a murine myeloma cell line until now considered to be the most positive for Xlr, mouse testis was found to transcribe abundant levels of this *Xlr*-related message (Figure 1B). This was in excess of 30-fold greater than V17C as determined by densitometry scanning of the autoradiogram (not shown). The testicular and the lymphocytic messages were of a very similar size, ~1 kb.

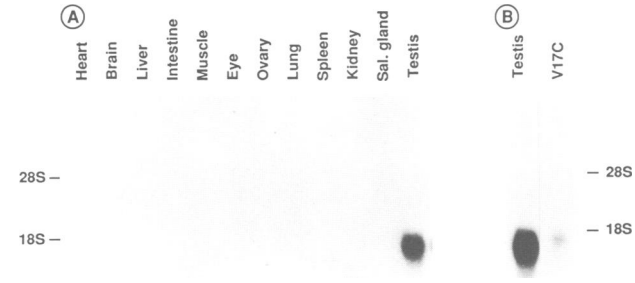


Fig. 1. Abundant expression of an *Xlr*-related message in adult mouse testis. (**A**) Northern blot hybridization of total RNA samples (10 μg) from various organs using the full-length cDNA pM1 probe (Siegel *et al.*, 1987). The final wash of the blot was in 0.1 × SSPE at 65°C. Reprobing of the blot with an actin probe indicated that equal amounts of RNA of each sample had been loaded on the gel (not shown). (**B**) The *Xlr* message is much more abundant in testis than in the V17C myeloma cell line.

The Xlr-related testicular transcript defines a novel functional member of the Xlr multigenic family

In order to determine the nucleotide sequence of this testicular message, overlapping fragments from testis cDNA were amplified in a stepwise manner using the polymerase chain reaction (PCR) protocol (Figure 2A). A unique sequence of 812 bp, called pT1, was obtained (Figure 2B). It showed a large segment of close homology (94% identity) with pM1 from position 389 up to its 3′ end, while the first 388 nucleotides were not related to any previously described *Xlr* sequence. The region of homology, however, was interrupted by three deletions. Notably, the first of these deletions (at position 539), which was also the largest one (Δ1, 153 bp), exactly corresponded to exons IV and V of the *Xlr* genomic sequences (Garchon *et al.*, 1989). The two other deletions (Δ2 and Δ3, at positions 570 and 777) were smaller (18 and 28 nucleotides, respectively) and were seen in exons VI and VIII of *Xlr* genomic variants. Although the absence of the sequence encoded by exons IV and V suggests that pT1 and *Xlr* could be derived from the same gene by alternative splicing, the finding that pT1 has 36 point mutations relative to *Xlr* supports the existence of two distinct genes.

The pT1 transcript has an ORF of 212 residues that begins with an initiator AUG codon at position 151 and ends at position 787. The corresponding protein has a predicted mol. wt close to 25 kDa. Study of its hydropathicity by the method of Kyte and Doolittle (1982) indicates that the protein is predominantly hydrophilic with the exception of two short hydrophobic stretches ($LWVI^{9}$ and $LLLL^{18}$) close to the N-terminus (not shown). In fact, these two tetrapeptides do not contribute to an effective signal peptide for a secretory pathway as the pT1 ORF corresponds to a nuclear protein (see below). They also do not fit the outline proposed for signal sequences (von Heijne, 1985). With respect to the nuclear localization of Xlr proteins, a short basic motif ($RKKI^{133}$) might serve as a nuclear localization signal (Garcia-Bustos *et al.*, 1991), perhaps in conjunction with potential casein kinase phosphorylation sites at position 102 (SELD) or 114 (THDD), like those documented for the SV40 large T antigen (Rihs *et al.*, 1991).

As expected from the comparison at the nucleotide sequence level, the pM1 and the pT1 proteins share two main segments of homology. The first one corresponds to the N-terminal acidic domain of pM1. In the pT1 protein, it is found in an intermediate position (residues 86–129). The variation between the segments (13/44 non-identical residues) results in a decrease in the negative charge relative to the pM1 protein (nine net negative charges in pT1 instead of 14 in pM1). The first 85 amino acids of pT1 do not match any segment of pM1 but still comprise an unusually high proportion of acidic residues (12 net negative charges).

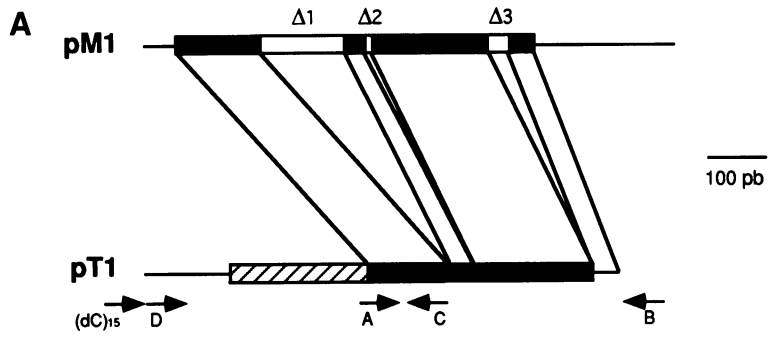
The second region of similarity between pM1 and pT1 maps to the C-terminal part of pT1 (residues 130–209) and shows 87.4% of residues identical to the corresponding segment of pM1 (residues 96–181). This latter region of pM1 could be aligned partly with human lamin C and mouse keratin II (Siegel *et al.*, 1987), two members of the intermediate filament superfamily of proteins well-known to display a coiled-coil structure. Analysis of pT1 with the 'Coiled-Coil' program (Lupas *et al.*, 1991) indicated a significant probability [$P(S) = 0.59$] for such a coiled-coil structure made of five heptads from residue 139 to residue 173. The putative coil presents a hydrophobic face which can interact with proteins having a similar α-helical structure (for review see Cohen and Parry, 1986).

Biochemical characterization of the pT1 gene product

Testicular cells were isolated from adult seminiferous tubules and were fractionated as described in Materials and methods. Immunoblot analysis with an anti-Xlr antibody (Figure 3) showed one predominant protein of apparent mol. wt very similar to that of the recombinant Xlr protein. It was found in the extractable nuclear fraction and, to a lesser but significant extent, in the insoluble nuclear fraction. A much weaker signal was seen in the cytoplasmic extract. It could correspond to the cytoplasmic form of the protein being synthesized or to some nuclear leakage during the fractionation procedure. Taken together, these results indicated that the pT1 cDNA identified a second functional member of the *Xlr* multigene family that presumably codes for a nuclear protein.

Tissue-specificity and developmental regulation of pT1 expression in male germ cells

By using the highly sensitive PCR technique for transcript amplification with the two primers B and D (see Figure 2A),



B

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pT1:1     ggacaacggttttttttcgattgaggagttgagcacggaagggtgcagttgtgaaggtgttcaccttctgagataac
 76       ctttaagtgataatcggctctgctcagggttgttggacagttaatcgagaagacaactgagcgcttaagagaaaa

pT1orf:1  M   S   I   K   K   L   W   V   I   P   K   D   G   Y   L   L   L   L   D
151       ATG TCT ATT AAG AAA CTG TGG GTG ATA CCA AAG GAT GGT TAC TTA TTA CTT CTT GAC

 20        Y   D   S   D   E   E   E   E   Q   A   H   S   E   V   K   R   P   A   F
209       TAT GAT TCA GAT GAA GAA GAA GAG CAG GCT CAT TCT GAA GTA AAG AGG CCA GCA TTT

 39        G   K   H   E   N   M   P   P   H   V   E   A   D   E   D   I   R   D   E
265       GGC AAA CAT GAG AAT ATG CCG CCT CAC GTA GAA GCA GAT GAA GAT ATA AGA GAT GAA

 58        Q   D   S   M   L   D   K   S   G   E   N*  V   S   F   S   E   E   W   Q
322       CAA GAC AGT ATG TTG GAT AAA TCT GGA GAA AAC GTA AGT TTC TCA GAG GAA TGG CAG

pM1orf:1                                          M   E   N   W   D   L   S   S   D   E
 77        R   F   A   R   S   V   E   T   P      M   E   N   W   N   L   L   S   G   E
379       CGT TTT GCA CGT TCT GTA GAG ACA CCA ATG GAA AAC TGG AAT TTG TTA AGT GGT GAA
pM1:50                  --  ---  tg-  ---  ---  a--  ---  ---  ---  ---  G-C  ---  -C-  ---  -A-  ---

 11        M   Q   D   G   N   A   P   E   L   D   V   I   E   E   H   N   P   V   T
 96        Q   Q   V   R   N*  A   S   E   L   D   L   M   E   V   Q   N   P   V   T
436       CAG CAA GTT AGG AAT GCT TCA GAA TTG GAC CTT ATG GAA GTA CAG AAT CCA GTA ACT
 97        AT- --- -A- G-- --- --- C-- --- --- --- G-- --T --- -A- --T --- --- --- ---

 30        R   D   D   E   N   A   N   P   E   E   V   V   G   D   T  ◊R   K   K   I
115        H   D   D   G   N   A   N   P   E   E   V   V   G   D   T   R   K   K   I
493       CAT GAT GAT GGG AAT GCA AAT CCT GAA GAA GTA GTT GGA GAT ACA CGA AAG AAG ATC
154       -G- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --Δ1--- --- --T

100       N   N   K   F   C   E   Q  ♦K   F   D   M   D   V   Q   K   F   N   E   E
134       N   N   K   L   C   E   Q   K   F   D   M   D   I   Q   K   F   N   E   E
550       AAC AAC AAA TTG TGT GAG CAG AAG TTT GAT ATG GAT ATA CAG AAA TTC AAT GAA GAA
364       --- --- --- --T --- --- ---Δ2-- --- --- --- --- G-C --- --- --- --- --- ---

125       Q   E   K   S   V   N   N   Y   Q   K   E   Q   Q   A   L   K   L   S   K
153       Q   E   K   S   V   N   N   Y   Q   K   E   Q   Q   A   L   K   L   S   E
607       CAA GAA AAA TCA GTG AAC AAT TAT CAA AAA GAA CAA CAA GCA TTA AAA CTG TCC GAA
439       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A--

144       C   S   Q   S   Q   T   L   E   A   I   K   D   M   H   E   N   Y   M   E
172       C   S   Q   S   P   T   M   E   A   I   E   D   M   H   E   K   S   M   E
664       TGT AGT CAG AGT CCG ACC ATG GAA GCA ATT GAA GAC ATG CAT GAG AAG TCC ATG GAG
496       --- --- --- --C -A- --- C-- --- --- --- A-- --- --- --- --A --C -A- --- ---

163       G   L   M   N   L   E   T   N   N   Y   N   M   L   F   D   V   D   G   E
191       G   L   M   N   M   E   T   N   N   Y   D   M   L   F   D   V   D   G   E
721       GGT TTG ATG AAC ATG GAG ACC AAC AAC TAC GAT ATG CTT TTT GAT GTA GAT GGT GAA
553       --- C-- --- --- T-- --- --- --- --- --- A-- --- --- --- --- --- --- --- ---Δ3

210       E   T   L
778       GAG ACA TTA tgaagcatactctgaagtactcttct
638       A-- --C -C- --------------------------
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Fig. 2. (**A**) Sequence relationship of the pT1 cDNA with the lymphoid-specific pM1 cDNA. Thin line: untranslated regions; thick bar: coding regions; closed region: homologous segments between pT1 and pM1; hatched region: pT1-specific sequence; open region: PM1-specific sequence; Δ1, Δ2 and Δ3 refer to segments of pM1 that are deleted in pT1. Arrows indicate the oligonucleotide primers used for amplifying the pT1 cDNA. Total RNA from testis was prepared in the BALB/c strain (where the pM1 sequence was previously obtained) and was copied into single-stranded cDNA. A first round of amplification was performed using specific A and B primers matching the pM1 sequence between its initiation and its stop codons. A fragment of 420 bp was obtained, smaller than the corresponding fragment normally amplified from lymphoid cell RNA (630 bp). Its nucleotide sequence was determined, allowing the design of a third primer, C, positioned immediately 3′ of primer A and pointing upstream. The cDNA was tailed with dG using terminal transferase and another round of amplification was performed using $(dC)_{15}$ and primer C in order to yield the 5′ end of the testis specific message, according to the anchored PCR scheme (Loh *et al.*, 1989). A 440 bp fragment was obtained and sequenced. To ascertain that the two overlapping amplified fragments originated from the same message, primer D matching the 5′ end of the cDNA sequence and primer B were used for amplification of the cDNA yielding an 812 bp fragment that was subcloned and sequenced. (**B**) Nucleotide sequence and predicted protein product of pT1 cDNA and comparison with pM1. Δ1, Δ2 and Δ3 refer to deletions in pT1 relative to pM1 (see text). ◊ and ♦ specify the existence of insertions in pM1 corresponding to Δ1 (51 residues) and Δ2 (six residues) respectively. Δ3 introduces a frameshift and a stop codon is read shortly after. The potential nuclear localization signal (RKKI[133]) is underlined. Asterisks indicate possible *N*-glycosylation sites. This sequence was deposited in EMBL data library under accession number X72697.

Fig. 3. Immunoblot detection of the pT1 gene product in testicular cells. Recombinant Xlr protein (1 ng, lanes 1 and 5), cytoplasmic (lanes 2 and 6), extractable nuclear (lanes 3 and 7) and insoluble nuclear (lanes 4 and 8) fractions (see Materials and methods) of testicular cells from an adult mouse were assayed by immunoblot with 1 μg/ml of pre-immune rabbit IgG (lanes 1–4) or affinity-purified anti-Xlr rabbit antibodies (lanes 5–8) followed by anti-rabbit IgG biotinylated antibodies and avidin–peroxidase.

expression of pT1 message at low levels in other organs than testis was ruled out (Figure 4A). In addition, and similarly to what had been observed in the lymphoid cell lineage, expression of pT1 appeared to be regulated in the course of testis differentiation. Initially, as early as 6 days after birth, low levels of message, detectable only by reverse transcription and PCR, were found (Figure 4B). Later, from 3 weeks of age, high levels of transcripts could be seen with the less sensitive Northern blotting technique (Figure 4C).

Germinal cells are known to enter the meiotic prophase 8–9 days after birth and then proceed through the successive stages of the first wave of meiosis in the next 12 days (Bellvé *et al.*, 1977; Goetz *et al.*, 1984). The temporal pattern of mRNA expression just described thus suggested that pT1 expression was related to spermatogenic events. Study of the pT1-encoded protein by immunofluorescence staining of testis sections, using an anti-Xlr monoclonal antibody, further supported this view. Whereas no cell was stained at birth (not shown), occasional cells inside some seminiferous tubules were faintly but specifically labelled at 8 days of age, when the first primary spermatocytes reach the pre-leptotene stage (Figure 5A). The staining of tubule basal lamina was non-specific and was also seen with the control MOPC21 monoclonal IgG1 protein (Figure 5B). At 12 days (Figure 5C) and 19 days (Figure 5D) of age, when the meiotic wave reaches the mid-pachytene and the diplotene stages respectively, the specific labelling gradually increased: more Xlr-positive cells were seen in all tubule cross-sections and, in addition, the intensity of the staining seemed much stronger than at day 8. Finally, in adult testis, spermatogenesis is completed with its characteristic luminal progression. Whereas the mature spermatids localized towards and within the tubule lumen, the Xlr-immunoreactive staining remained exclusively in the basal meiotic compartment (Figure 5E).

Altogether, this temporal and spatial pattern of expression of the pT1 gene product closely paralleled the onset and the progression of the prophase of the first meiotic division. Simultaneous examination of stained cells under phase contrast indicated that the immunofluorescence signal was restricted to the germ cells and that it localized to their nuclei (not shown). Moreover, from day 12 after birth, two patterns

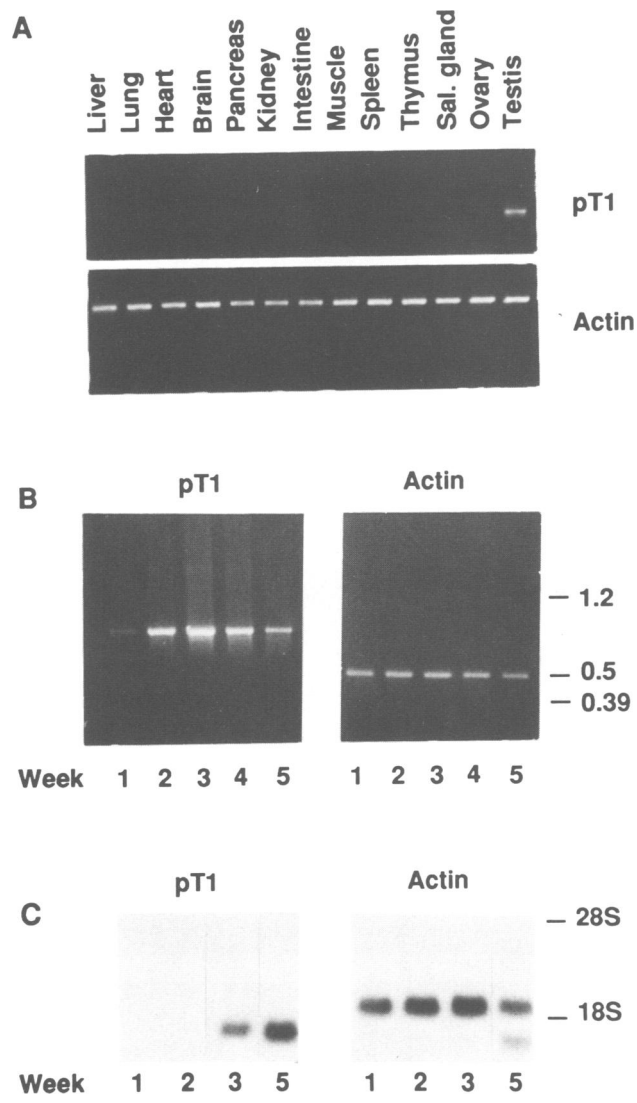

Fig. 4. (**A**) Tissue specificity of pT1 gene expression: total RNA from various organs from adult mice were reverse-transcribed and amplified using the PCR protocol with B and D primers (see Figure 2A). Amplification with β-actin-specific primers served as a control. (**B**) Ontogeny of pT1 message expression in testis detected by PCR:pT1 message was PCR-amplified from total RNA obtained at the indicated time after birth. (**C**) Ontogeny of pT1 message expression in testis was detected by Northern blotting using the pT1 cDNA probe and, for actin, the blot was rehybridized with an actin probe.

of nuclear staining, a diffuse one and a spotted one, could be seen and, therefore, were analysed in further detail.

Stage-specific subnuclear localization of the pT1 gene product during the meiotic prophase and its mapping to the XY body

Examination of testis sections processed with the biotin–avidin immunoperoxidase method and counterstained with haematoxylin unambiguously confirmed the nuclear localization of the pT1 protein (Figure 6A). Immediately striking, was the dual aspect of nuclear staining with the anti-Xlr antibody: in addition to the pattern already described in lymphoid cells where the whole nucleus was stained, a spotted pattern could also be visualized either separately or together with the first pattern. Cells expressing pT1 were present in all tubule sections. Only primary spermatocytes

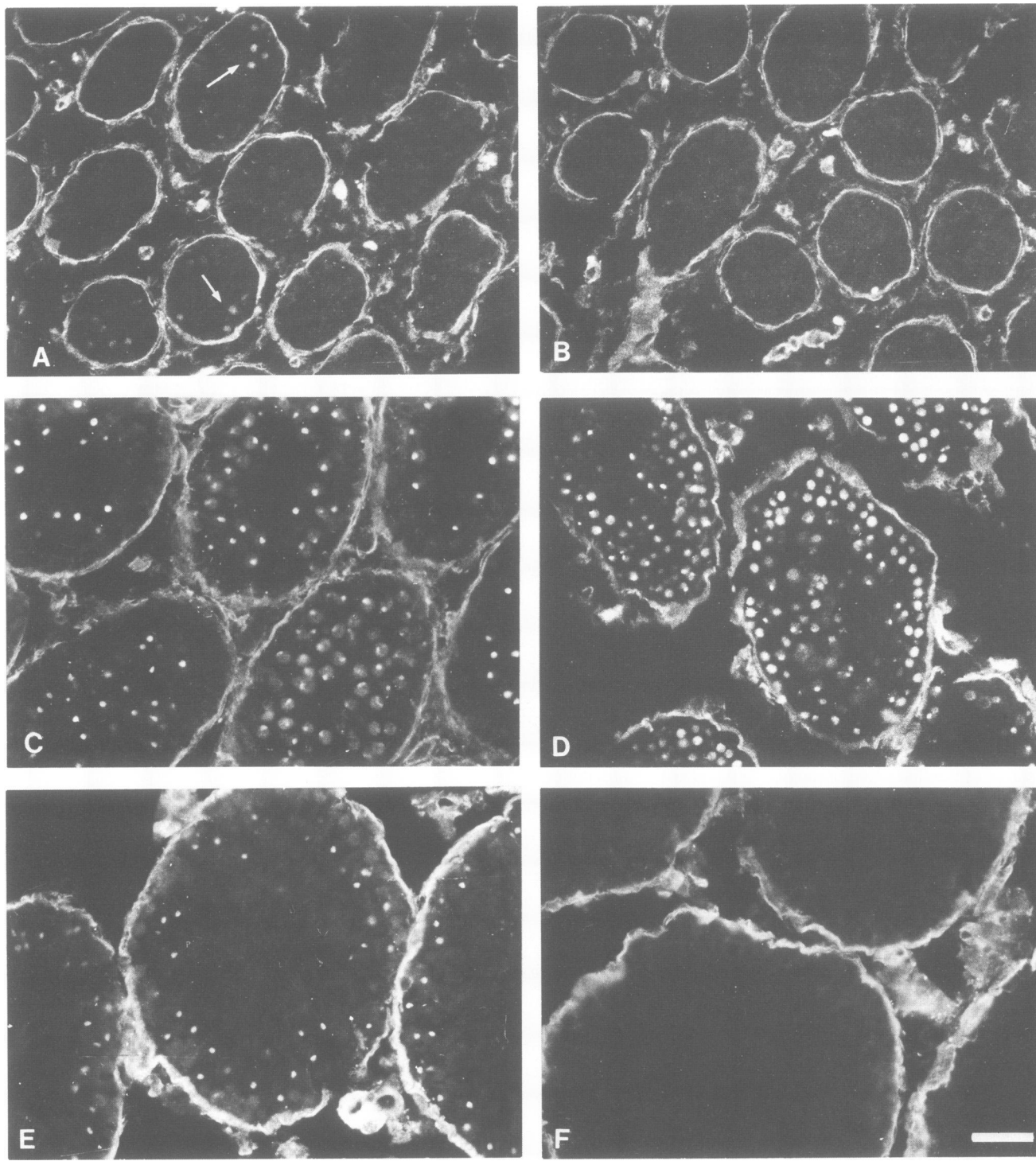

Fig. 5. Ontogeny of pT1 protein expression in developing testis. Immunofluorescence detection of the pT1 ORF on testis sections using anti-Xlr RIK2D3 monoclonal antibody (**A**, **C**, **D** and **E**) or control MOPC21 myeloma protein (**B** and **F**), at 8 days (A and B), 12 days (C), 19 days (D) and 10 weeks (E and F) of age when the first spermatogenic wave reaches the pre-leptotene, the mid-pachytene and the diplotene stages respectively. Arrows in (A) point to scarce faintly positive cells. Note the growth of seminiferous tubules. Magnification is the same for all sections. Bar = 20 μm.

were labelled; secondary spermatocytes and post-meiotic cells were free of any staining.

Spermatogenesis in rodents initiates segmentally around the circumference of the tubules and then progresses towards their lumen. Simultaneously, its initiation propagates along the length of the tubules as a wave. In mice, the whole process takes 35 days, of which 12 days correspond to meiosis, and re-initiates every 12 days. As a result, layers of cells at various points in the spermatogenic pathway reproducibly combine in specific associations which have been classified into 12 histologic stages (Oakberg, 1956). Examination of testis sections and staging of the spermatogenic cycle permitted a precise identification of the meiotic steps associated with pT1 expression. The labelling was first observed in pre-leptotene spermatocytes as a faint staining of the whole nuclear content (Figure 6D). This nuclear

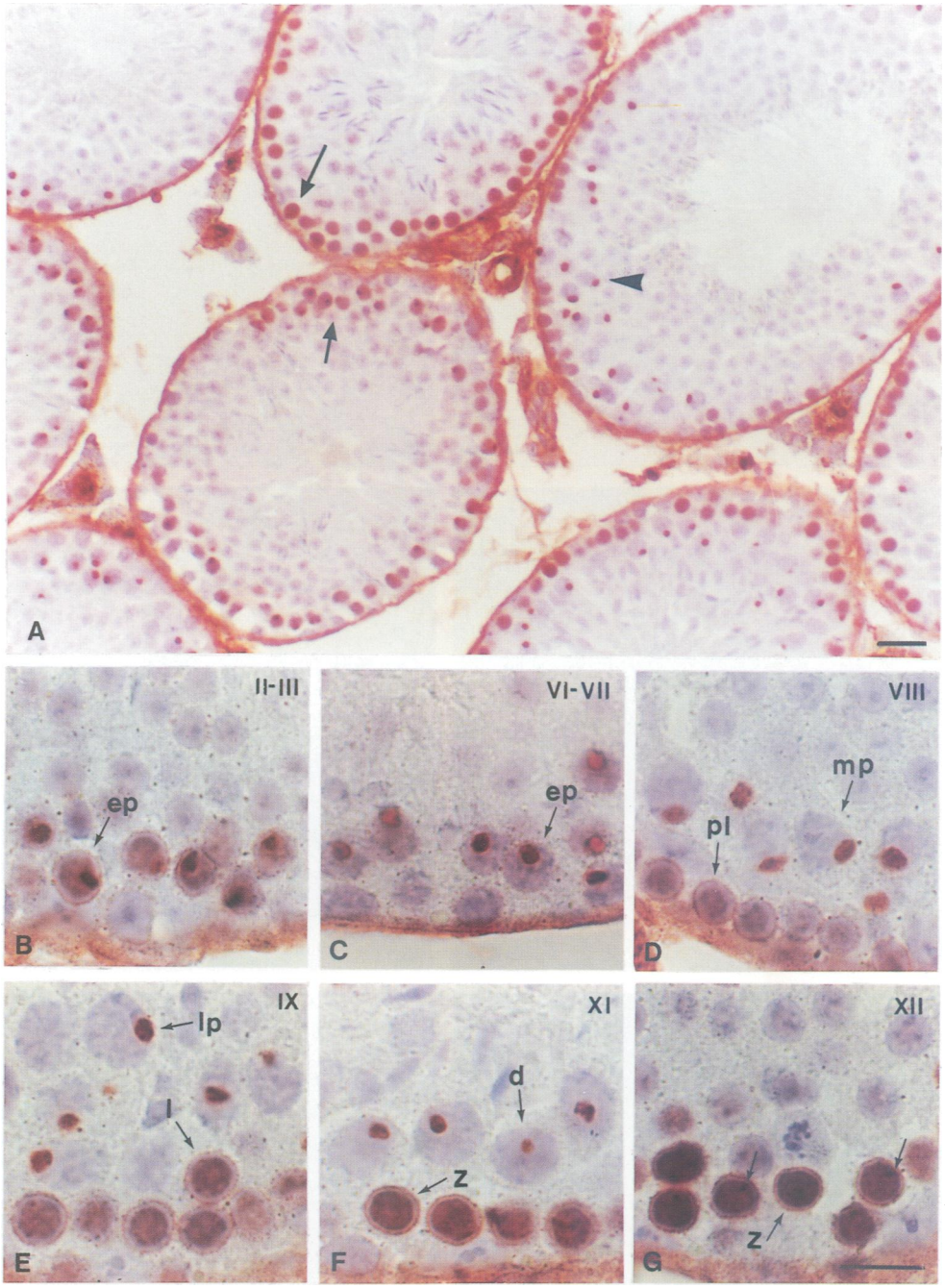

Fig. 6. Nuclear compartmentalization and stage-specificity of pT1 protein expression in primary spermatocytes. Testis sections of an adult mouse were immunolabelled with RIK2D3 anti-Xlr monoclonal antibody and the avidin–biotin peroxidase system. (**A**) Overview of several seminiferous tubules showing the three patterns of pT1 staining affecting either the whole nucleus (arrow) of the sex vesicle (arrowhead) or both (short arrow). Only cells undergoing meiosis in the basal compartment were labelled. Bar = 20 μm. (**B–G**) Detailed characterization of the stage-specificity of pT1 expression revealed that the whole nucleus staining could be seen from pre-leptotene (D), reached a maximum at zygotene (F and G) and disappeared at mid-pachytene (D) while the sex vesicle was stained from zygotene (G, arrow) until diplotene (F). Roman numerals refer to the stage of spermatogenesis (Oakberg, 1956; Russell *et al.*, 1990). pl: pre-leptotene; l: leptotene; z: zygotene; ep: early pachytene; mp: mid-pachytene; lp: late pachytene; d: diplotene. Bar = 15 μm.

staining increased in intensity until the zygotene stage (Figure 6E–G). At this latter point, a differentially stained nuclear subregion appeared (Figure 6G, arrow). In early-pachytene spermatocytes, the labelling of the nucleus as a whole became fainter whereas the subregion could then be clearly distinguished (Figure 6B and C). In mid-pachytene spermatocytes (Figure 6D) and at later steps, only this nuclear domain was stained with anti-Xlr antibodies. It appeared as an intense spot with an ovoid shape, 3 μm long and 2 μm wide. In diplotene cells, it moved back toward the centre of the nucleus while it became fainter (Figure 6F), eventually disappearing before metaphase-1 (Figure 6G).

The morphological characteristics of the pT1-positive spot and the temporal pattern of its appearance (from zygotene to diplotene) exactly fit those of the XY body (Solari, 1974). The localization of pT1 in the XY body was confirmed by immuno-electron microscopy (Figure 7). From early pachytene, the XY domain is connected to the nuclear envelope.

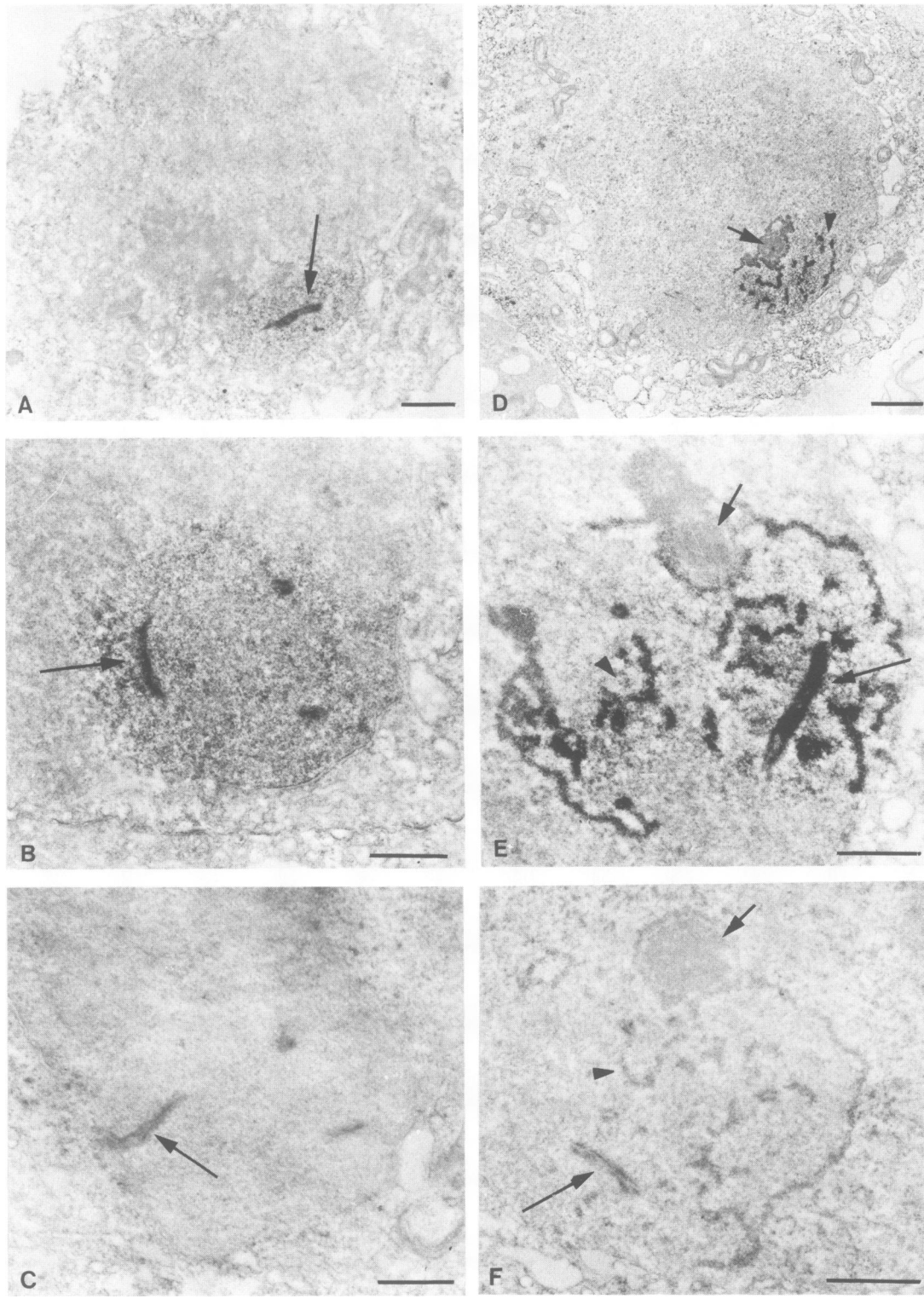

Fig. 7. Ultrastructural localization of Xmr in the XY body, at early (**A–C**) and late (**D–F**) pachytene stages by immunoperoxidase staining, using RIK2D3 anti-Xlr monoclonal antibody (A, B, D and E) or control IgG1 (C and F) as primary reagent. The axes of sex chromosomes (long arrow) were specifically labelled (A, B and E). At late pachytene, the XY body was associated with the granular component of the nucleolus (arrowhead), also labelled, and by the unstained round body (short arrow). In A and D the bar represents 1 μm; in B, C, E and F the bar represents 0.5 μm.

The XY body appears lighter and more homogeneous than the autosomal part of the nucleus. The axes of sex chromosomes were condensed and were strongly labelled by an anti-Xlr monoclonal antibody (Figure 7A). At a higher magnification, the pT1 labelling of the axes could be distinctly seen (Figure 7B). The remaining part of the XY domain was also stained, but more weakly and heterogeneously, especially in the immediate vicinity of the axes. The nucleolus develops from mid-pachytene, reaching its maximum size at late pachytene, when it covers the inner side of the XY body (Figure 7D). The axes were still intensely labelled (Figure 7E), again with a diffuse labelling

in their vicinity. In addition, a trabecular pattern of staining was prominent. It was very similar to the invaginations of the granular part of the nucleolus into the XY area (Solari, 1969; Knibiehler *et al.*, 1981). By contrast, the round body was not stained, and nor could specific labelling be detected in the region of the nucleus that contains autosomes, possibly due to the technical conditions of sample preparation and to an alteration of antigenic determinants.

In mutant mice bearing the reciprocal t(X;16) Searle's translocation, spermatogenesis is blocked at the time of, or early after, the first meiotic division (Searle, 1989). The distinctive structure of sex chromatin in primary spermatocytes extends to the translocated autosome and the XY body is rather enlarged in contrast to the nucleolus which hardly develops (Solari, 1971). Its shape is also altered and often shows an extension across the nucleus. Staining of testis sections from such mice with RIK2D3 anti-Xlr monoclonal antibody showed pT1-labelled spots with a conserved size but of heterogeneous shape when compared with those seen in normal mice (Figure 8). This observation further supported the association of the pT1 gene product with the XY body.

Discussion

In this work, we have characterized a second functional member of the *Xlr* gene family. We found that this gene is precisely expressed during the prophase of the first meiotic division in male germ cells where it appears to be a component of the XY body. Accordingly, we propose the name *Xmr* (Xlr-related, meiosis regulated) for this gene. Although a series of loci that are expressed both during and after meiosis or even throughout spermatogenesis, have previously been reported (Wolgemuth and Watrin, 1991), few genes have been identified to date in specific association with meiotic prophase in mammals (Markose and Rao, 1989; Cunliffe *et al.*, 1990; Grimes *et al.*, 1990; Keshet *et al.*, 1990; Meuwissen *et al.*, 1992; Ruppert *et al.*, 1992; Trasler *et al.*, 1992). Similarly, few prophase-specific proteins have been described (Stern, 1986; Behal *et al.*, 1987; Moens *et al.*, 1987; Smith and Benavente, 1992a,b). The *Xmr* gene product thus provides a new tool that will be most useful for the study of meiotic nuclei as it has been defined both at the DNA and at the protein levels.

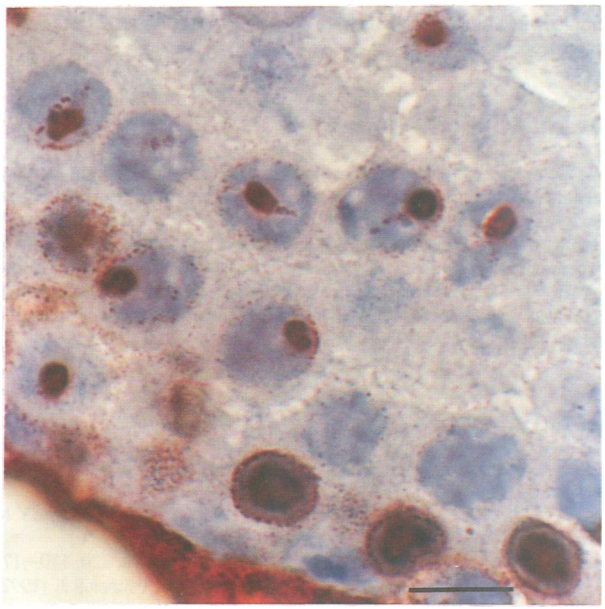

Fig. 8. Immunoperoxidase staining of a testis section from an adult mouse bearing Searle's translocation (t[X;16]) with an anti-Xlr monoclonal antibody. Note the highly heterogeneous shape of the XY body labelling. Bar = 12 μm.

Our most remarkable finding was the subnuclear localization of Xmr in the XY body. Presently, little is known about the molecular structure of this nuclear domain, which is likely to play an essential role in the specific behaviour of sex chromosomes during meiosis. Immuno-electron microscopy revealed that, within the XY body, Xmr was associated with the axes of sex chromosomes during most of the pachytene stage. Recently, a 40 kDa protein exclusively associated with the X and Y axes, termed XY40, was identified in rat spermatocytes using a monoclonal antibody (Smith and Benavente, 1992b). Xmr is likely to be distinct from XY40 as it was not exclusively associated with the axes of sex chromosomes in the XY body and as it was also localized in the autosomal part of the nucleus. This latter localization could be only transient and represent the protein *en route* to the XY body after its synthesis in the cytoplasm. Alternatively, Xmr could have a similar function in the autosomal part of the nucleus and in the XY domain, the difference in the timing of their expression being related to the allocyclic condensation of X and Y chromosomes. The latter possibility is also supported by the finding of an Xmr-cross-reactive protein in pachytene oocytes, in fetal ovaries, at days 16–17 of gestation (our unpublished data); this is another distinctive feature as XY40 is male-specific.

Besides the axes of sex chromosomes, additional structures were stained in the XY domain, especially at late pachytene where the Xmr pattern was very similar to invaginations of the granular component of the nucleolus (Solari, 1969; Knibiehler *et al.*, 1981). The close association of nucleolar components with the XY domain is intriguing and has even led to some confusion concerning the actual origin of the XY domain. Its significance is presently unknown.

The developmentally regulated expression of Xlr and Xmr proteins points to the existence of a pathway of differentiation common to the lymphoid and germ cell lineages. Most interestingly, we have recently observed high levels of expression of an Xlr protein in immature fetal thymocytes (B.Allenet, D.Escalier and H.-J.Garchon, manuscript in preparation). Xlr and Xmr therefore happen to be expressed at stages of differentiation in both cell lineages that, characteristically, are associated with DNA rearrangements.

```
INNKLCEQKFDMDIQKFNEEQEKSVNNYQKEQQALKLSECSQSPTMEAIEDMHEKSMEGLMNMETNNYDMLFDVDGE
=-= =       = - = -=   ==   = - = -= - = =   -  -   - -=  =-   =   - -===-= --
IENQLDKVSTAM.LSKGDEKKTKSDSSY.R.Q.VL.VEEISRYNSKIT.RHVTNKQHETEKSMRCTQ.EMLFNVGSQ
```

Fig. 9. Sequence similarity between the Xmr ORF and the MER2 protein from *S.cerevisiae*. Comparison was performed with the algorithm of Goad and Kanehisa (1982).

The analysis of the primary structures of Xlr and Xmr, showing that they include an acidic region, in a different position in each protein, and a putative coiled-coil domain, strengthens the idea that they could belong to the family of modular acidic activators of transcription. Comparison of the Xmr amino acid sequence with a set of 40 meiosis-associated proteins in the Swiss-Prot data library, using the programs of Smith and Waterman (1981) or Goad and Kanehisa (1982), revealed a highly relevant similarity with the *MER2* gene product from *Saccharomyces cerevisiae* (Engebrecht *et al.*, 1990). It is related to the C-terminal part of Xmr with 26% (20/77) identical residues (Figure 9). The *MER2* gene product is essential for meiosis and appears to be involved in gene conversion or in the formation of synaptonemal complexes. The correlation of the time course of Xmr expression with the cycle of chromatin condensation also suggests that Xmr could be involved in DNA metabolism of meiotic chromosomes. These comparative data provide a working hypothesis that can be tested by functional studies.

Materials and methods

RNA isolation and Northern blotting

RNA samples were isolated using the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979). Aliquots were electrophoresed through a 1.2% agarose gel in formaldehyde–MOPS buffer and blotted onto nylon membrane using established protocols (Sambrook *et al.*, 1989). Hybridization was performed in Church solution (Church and Gilbert, 1984) with the pM1 full-length cDNA probe labelled with [α-^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1983). Filters were washed at a final stringency of 0.1 × SSPE at 65°C.

Characterization of the Xmr cDNA

Total RNA from testis was prepared in a 10 week old BALB/c mouse and was reverse-transcribed using Mu-MLV reverse transcriptase (Gibco-BRL). The cDNA was first amplified with the Taq DNA polymerase (Saiki *et al.*, 1985), using two gene-specific primers, A (5′-CTTGAGAGACAACAA-TGGAAAAC-3′) and B (5′-AGTCTGAAGATGGGAAACTAGAAG-3′) matching respectively the 5′ end and the 3′ end of the coding region of the pM1 cDNA (Figure 2A). The 5′ end of the cDNA was then amplified following the anchored-PCR protocol (Loh *et al.*, 1989). Briefly, the single-stranded cDNA was tailed with dGTP using terminal-deoxynucleotidyl transferase (Deng and Wu, 1983). The tailed cDNA was then amplified using $(dC)_{15}$ as the non-specific 5′ primer and the *Xlr*-specific primer C (5′-TAACTTGCTGTTCACCACTTAACAAATT-3′). Finally, the cDNA was amplified with the two specific primers D (5′-ATTGAGGAGTTGAGC-ACGGAA-3′) and B to ensure that the two primary PCR fragments originated from the same cDNA. The PCR products were subcloned into the *Eco*RV site of the BlueScript(KS+) plasmid (Stratagene). Double-stranded DNA of recombinant plasmids was sequenced using the dideoxynucleotide chain termination technique (Sanger *et al.*, 1977) and the modified T7 DNA polymerase (Tabor and Richardson, 1987). Sequences of both strands of 16 clones obtained from two independent amplifications were determined in BALB/c (11 clones) and C57BL/6J (five clones) strains.

Anti-Xlr antibodies

Recombinant Xlr protein and affinity-purified rabbit polyclonal anti-Xlr antibodies were prepared as described previously (Garchon and Davis, 1989). Monoclonal antibodies were produced after immunization of a 9 week old BALB/c female mouse with purified recombinant Xlr protein. Growing hybridomas were screened by solid-phase ELISA. One clone RIK2D3 (IgG1κ) was found suitable for immunolabelling on tissue sections and was expanded for further study.

Cell fractionation and immunoblot assay

Testes of a 6 week old C57BL/6 mouse were decapsulated and dissociated by mild collagenase digestion (Silver *et al.*, 1979). A single cell suspension was obtained by treatment with trypsin. Cells were rinsed in cold PBS and lysed in lysis buffer (10 mM HEPES pH 7.6, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $ZnSO_4$, 0.4% Nonidet P-40) as described by Garchon and Davis (1989). The cytoplasmic fraction was harvested. Nuclei were centrifuged through a 30% sucrose cushion, washed once, resuspended in extraction buffer (10 mM HEPES pH 7.6, 0.5 M NaCl, 10 mM EDTA) and pelleted again. The supernatant (extractable nuclear fraction) was harvested and mixed with an equal volume of 2× reducing sample buffer (Laemmli, 1970). The pellet (insoluble nuclear fraction) was solubilized in 1× reducing sample buffer. Approximately 1 μg of each fraction was run on a denaturing SDS–12% polyacrylamide gel and blotted onto nitrocellulose (Towbin and Gordon, 1984). After saturation with 5% non-fat milk powder and 0.05% Tween-20, membranes were incubated with 1 μg/ml of affinity-purified anti-Xlr rabbit antibodies or of pre-immune IgG followed by goat anti-rabbit IgG biotinylated antibodies and avidin–peroxidase. Enzymatic activity was detected with ECL substrate (Amersham).

Immunohistochemical labelling

Testes were decapsulated, fixed for 3 h in 4% paraformaldehyde in PBS (phosphate-buffered saline) and then incubated in sucrose solutions of increasing concentration (12%, 15% and 18%) before freezing and sectioning. For immunofluorescence labelling, sections (4 μm) were incubated with the RIK2D3 monoclonal antibody or with control MOPC21 IgG1κ (5 μg/ml). Texas Red-conjugated goat $F(ab)'_2$ antibodies to mouse IgG (Biosys) were used as the second step reagent. Alternatively, immunohistochemical labelling was performed with the three-step immunoperoxidase technique using the biotin–avidin system (Vector Laboratories). Amino-ethyl-carbazole was used as the chromogen. Sections were counterstained with Harris haematoxylin and mounted in aqueous medium (Glycergel, Dakopatts). Stages of mouse spermatogenesis and of primary spermatocyte differentiation were identified according to Oakberg (1956) and Russell *et al.* (1990).

Immuno-electron microscopy

Germ cells from male mice were isolated as above. After fixation for 90 min in 4% paraformaldehyde, cells were permeabilized with 0.05% saponin in PBS and incubated overnight in 5% normal horse serum. Cells were then incubated with 7 μg/ml of either RIK2D3 monoclonal antibody or control IgG1. Peroxidase-conjugated anti-mouse antibody (Biosys) was used as the second step reagent and, after an additional 30 min fixation period in 1% glutaraldehyde, diaminobenzidine was used as the chromogen. Post-fixation was done with 1% OsO_4 and processing for electron microscopy was performed in Araldite. Uranyl acetate and lead citrate counterstaining were omitted and ultrathin sections were observed on a Philips EM-300 electron microscope.

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