Transcription of meiotic cell cycle and terminal differentiation genes depends on a conserved chromatin associated protein, whose nuclear localisation is regulated

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Accepted 10 October; published on WWW 14 November 2000

SUMMARY

The Drosophila always early (aly) gene coordinately regulates meiotic cell cycle progression and terminal differentiation during male gametogenesis. aly is required for transcription of key G2-M cell cycle control genes and of spermatid differentiation genes, and for maintenance of normal chromatin structure in primary spermatocytes. We show that aly encodes a homologue of the Caenorhabditis elegans gene lin-9, a negative regulator of vulval development that acts in the same SynMuvB genetic pathway as the LIN-35 Rb-like protein. The aly gene family is conserved from plants to humans. Aly protein is both cytoplasmic and nuclear in early primary spermatocytes, then resolves to a chromatin-associated pattern. It remains cytoplasmic in a loss-of-function missense allele, suggesting that nuclear localisation is critical for Aly function, and that other factors may alter Aly activity by controlling its subcellular localisation. MAPK activation occurs normally in aly mutant testes. Therefore aly, and by inference lin-9, act in parallel to, or downstream of, activation of MAPK by the RTK-Ras signalling pathway. We favour a model where aly may regulate cell cycle progression and terminal differentiation during male gametogenesis by regulating chromatin conformation in primary spermatocytes.

Key words: Meiosis, SynMuv, Spermatogenesis, Differentiation, Chromatin, Rb, Aly, Drosophila

INTRODUCTION

Normal development of multicellular organisms relies on the integration of extracellular signals with cell intrinsic factors to co-ordinate the cell division cycle with morphogenetic differentiation events. Thus, the interpretation of extracellular signals (e.g. growth factors) depends on the intrinsic responsiveness of the cells (e.g. receptor expression or chromatin structure), and so a single signal can lead to different developmental decisions. For example, constitutively active Ras promotes continuous cell proliferation and contributes to tumourigenesis by activation of mitogen-activated protein (MAP) kinase (reviewed in Barbacid, 1987). In a different developmental context, a specific signalling cascade through the RTK sevenless and Ras to MAP kinase specifies, and induces terminal differentiation of, the R7 photoreceptor cell in the Drosophila eye. This is mediated by alteration of the activity of both transcriptional activators and repressors (Brunner et al., 1994; Lai and Rubin, 1992; Zeidler et al., 1996). Chromatin structural changes may play an important role in terminal differentiation, providing a mechanism by which to globally downregulate genes not required for the particular differentiation pathway, while still allowing activated transcription of specific genes. In C. elegans the SynMuv A and B genetic pathways negatively regulate the specification of vulval cell fate (Ferguson and Horvitz, 1989). Recent research showed that some members of the SynMuv pathways are involved in chromatin structure, including components or regulators of the nucleosome remodelling and histone deacetylase (NURD) complex and the tumour suppressor protein Rb (Hsieh et al., 1999; Lu and Horvitz, 1998; Solari and Ahringer, 2000). Thus, the balance between activity of a chromatin remodelling complex and activity of MAP kinase modulates the cell fate decision to initiate either the vulval or the hypodermal terminal differentiation pathway. Cell cycle progression and cellular differentiation must be closely coordinated during the formation of male gametes. In Drosophila, this coordination of the specialised cell cycle of meiosis with the terminal differentiation programme of spermiogenesis is mediated by the activity of the meiotic arrest genes. The initiation of spermatid differentiation is independent of the meiotic divisions. However, mutation of any one of eight meiotic arrest genes causes arrest of both processes (Lin et al., 1996; H. W.-C. and M. T. F., unpublished observations). The meiotic arrest genes regulate entry into the meiotic divisions by controlling the expression of key proteins required for cell cycle progression from G2-Meiosis I (White-Cooper et al., 1998). These include Cyclin B, a component of
the major Cdk/Cyclin complex involved in the G2-meiosis I transition, and Twine, which dephosphorylates and thereby activates Cdc2 kinase (Alphay et al., 1992; Gautier et al., 1991; White-Cooper et al., 1993).

The meiotic arrest gene *always early* (*aly*) appears to play a crucial role at the head of the pathway controlling transcription of both cell cycle and differentiation genes. *aly* is required for expression of both *cyclin B* and *twine* in primary spermatocytes. In addition to regulating the expression of cell cycle genes, *aly* also regulates spermatid differentiation by controlling transcription in primary spermatocytes of a suite of spermatid differentiation genes (White-Cooper et al., 1998). In addition to its role in transcriptional activation, *aly* is required for maintenance of normal chromatin structure in primary spermatocytes. Here we show that *aly* is a member of a protein family conserved from plants to humans, and is homologous to the *C. elegans* SynMuV-B gene *lin-9* (Beitel et al., 2000). *lin-9* acts in the same genetic pathway as the Rb homologue *lin-35*, and negatively regulates vulval induction by antagonising a RTK-Ras-MAF signal transduction cascade. We show *aly* is not required for the activation of MAP kinase in testes. Localisation of Aly protein in spermatocytes is regulated according to their developmental state, changing from an early nuclear and cytoplasmic distribution to later become chromatin associated. The cytoplasmic localisation of a mutant form of Aly protein suggests that the normal function of *Aly* protein depends on its nuclear localisation. The behaviour of Aly protein, its homology to the *C. elegans* SynMuV-B gene *lin-9*, and the abnormal chromatin structure in *aly* mutant spermatocytes suggest that *aly* may control transcription of cell cycle and terminal differentiation genes by regulating a chromatin remodelling complex.

**MATERIALS AND METHODS**

Stock descriptions and maintenance

Fly stocks were maintained on standard cornmeal yeast agar medium at 25°C unless otherwise stated. Balancer chromosomes and marker mutations are described in (Lindsley and Zimm, 1992), and are also available on FlyBase (http://flybase.bio.indiana.edu). *yw; P[w +] BGT-SO19* is a homozygous viable insertion in 63A4-6 and was obtained from Berkley Drosophila Genome Project. Four new *aly* alleles, *aly*3-402, *aly*3-407, *aly*3-1991, and *aly*3-1904 were obtained from a collection of male sterile mutations identified by B. Wakimoto and D. Lindsley from the C. Zuker collection of viable EMS mutagenised lines.

Recombination mapping

*w; ru* *aly* h can/ th st sa1 / TM3 flies were crossed to *yw; P[w +] BGT-SO19*, and the virgin female non-balanced progeny crossed to *w; ru th h st cu or ru h st cu e Pr ca* males. *F2 ru w + h* or *ru w + h* males were crossed to *w; aly* red e / TM3 females. Male *Sb*+, *Pr*+ progeny of this cross were scored for the *aly* phenotype by testis dissection. Of 208 recombinants in the *ru-h* region, one recombinant separated *aly* from *P[w +] BGT-SO19*, and indicated that *aly* lies distal to the *P[w +] BGT-SO19* insertion site.

Generation of deficiencies by male recombination

*yw; + / CyO Δ2-3; y* red e / TM6C males were crossed to *yw; P[w +] BGT-SO19* females to generate male progeny *yw/ y; + / CyO Δ2-3; y* red e / *P[w +] BGT-SO19* in which recombination at the *P* element could occur. These were crossed to *yw; TM3 (not y*+) / TM6B females.

Recombinations resulting in a *y* + *e* + chromosome could potentially have a deletion or insertion distal to the *P* element. Of approximately 35,000 F2 flies, 33 independent *y* + *e* + chromosomes were recovered by crossing to *w; aly* red e / TM3. Of these 13 had lost, and 20 had retained, the *P[w +]* element. All of the recombinant chromosomes were homozygous viable except one, which had a transposition of the *P* element to elsewhere on the third chromosome, and all fully complemented the *aly*2 phenotype. Southern blots of DNA from homozygous recombinant flies were probed with whole *λ* phage inserts from the *aly* region (see below). Two recombinants had deletions large enough to change the restriction fragment pattern. The larger deletion (4Q1.2), still had a *P*-element at the original insertion site, but lacked sequences distal to this site. The flanking DNA from this element was cloned by plasmid rescue and mapped to the phage walk. The deletion in this stock extends 22 kb distal to the original insertion site (Fig. 1).

**Molecular cloning**

Using 32P labelled probes generated by random priming with the Amersham Megaprime kit, DNA flanking the pl.wB element insertion *BGT-SO19* was cloned by plasmid rescue and used to probe a phage genomic library in λEMBL3 (Tamkun et al., 1992). Contigs were assembled by cross hybridisation and restriction mapping. The orientation of the cloned DNA relative to the chromosome was determined by in situ hybridisation to polytene chromosomes. The distal-most fragment was used to extend the walk, screening both the Tamkun library and a second genomic phage library in Charon 4 (Maniatis et al., 1978).

Five cDNAs were isolated from 1×106 plaques of the Hazelrigg λ-ZAP testis cDNA library screened with a probe covering most of –24 to –42 on the walk. Three (0.85 kb) were essentially identical, apart from slight differences in the length of the poly-A tail. One was longer (1.2 kb), but chimaeric at the 5′ end; the longest cDNA was 1.5 kb and contained DNA from two exons.

5′ RACE was carried out using the Gibco 5′ RACE kit, following the manufacturers instructions. PCR primers were designed so that the 1.9 kb transcript should produce a product of 750 bp. Nested primers were designed to re-amplify the product. The product was subcloned into pBluescript SK+ using T4 polymerase exonuclease digestion of the PCR products, and two of the subclones were sequenced. The bulk PCR product was also sequenced.

**DNA sequencing and PCR**

Cycle sequencing was carried out using the Big-Dye sequencing kit (ABI) according to the manufacturer’s instructions. The reactions were run on an ABI 377 automated sequencer. Sequences were assembled into contigs and aligned using Sequencher software (GeneCodes Corp). Oligonucleotides were synthesised by MWG Biotech. PCR was carried out using Qiagen Taq polymerase in a Biometra T3 thermal cycler.

To sequence mutant alleles, five overlapping products of about 450 bp that covered the entire open reading frame were generated by PCR, using genomic DNA from homozygous mutant flies as template (0.2 flies per PCR). PCR products were gel purified with the Qiaquick gel purification kit (Qiagen), and each bulk product was sequenced from both ends using the same primers as were used for the initial amplification.

**RNA methods**

Total RNA was extracted from whole males using RNAzol (Tel-Test Inc.) or Trizol (Gibco-BRL). Poly A+ RNA was selected in batch on oligo dT-cellulose beads. Approximately 500 ng (50 males worth) of poly A+ RNA was loaded in each lane of a 1.2% agarose gel containing formaldehyde. After running, the RNA was partially hydrolysed by incubating the gel in 0.05 M NaOH for 20 minutes, washed in water and transferred to Zeta-probe GT nylon membrane (BioRad) in 10× SSC. Filters were pre-hybridised at 65°C for 4 hours.
in buffer containing 4× SSPE, 1× Denhardt’s reagent, 1% SDS and 0.35 mg/ml salmon sperm DNA. Hybridisation was carried out overnight at 65°C in the above buffer containing 10% dextran sulphate. Filters were washed at 65°C in 0.2× SSC, 0.1% SDS.

RNA in situ hybridisation was carried out as previously described (White-Cooper et al., 1998), using sense and antisense RNA probes generated from a cDNA clone that contained the 3′ 850 bp of the transcript.

**Antibody production**

A 744 bp fragment of the aly C-terminus (bases 1142-1886 on Fig. 1) was PCR amplified from cloned cDNA, ligated into pQE-30 (Qiagen) after digestion with BamHI and Sall, sites for which were engineered into the PCR primers, and transformed into XL-1 blue. 6-His tagged fusion protein was purified after induction with IPTG using Ni/NTA agarose beads (Qiagen). Two rabbits were each immunised with a first injection dose of 500 μg, and four additional boosts of 250 μg of purified protein.

**Western blotting**

Protein extracts were prepared by dissecting testes in testis buffer (187 mM KCl, 47 mM NaCl, 10 mM Tris pH 6.8), or modified testis buffer plus phosphatase inhibitors (10 mM Tris-Cl pH 6.8, 180 mM KCl, 50 mM NaF, 1 mM NaVO₄, 10 mM β-glycerophosphate) for the dp-ERK and total ERK experiments, transferring to a 1.5 ml eppendorf tube, adding SDS sample buffer and boiling immediately for 5 minutes. Samples were run on 8% or 12% acrylamide gels, then transferred to Immobilon-P membrane (Millipore). Equivalence of protein loading between lanes was judged by Ponceau-S staining of the membrane after transfer. Membranes were blocked in 5% non-fat milk in PBS 0.1% Tween-20. Primary antibodies: the rabbit anti-Aly antibody was used at 1:12000, mouse monoclonal anti Dp-ERK (clone MAPK-YT [M8159], Sigma) at 1:750, and rabbit anti-ERK1 and ERK2 (Promega) at 1:2000. Secondary antibodies: goat anti-rabbit HRP and horse anti-mouse HRP (Vector Labs) were used at 1:10000. Signals were detected using the Pierce Supersignal kit.

**Immunofluorescence and immunohistochemistry**

For both immunofluorescence and immunohistochemistry, only males less than 1 day old were used. Indirect immunofluorescence on squashed testes was carried out essentially as previously described (White-Cooper et al., 1993), using the polyclonal anti-Aly antibody (Vector) secondary antibodies were diluted 1:4000 and 1:2000 respectively in PBS-Tx 5% FCS. Extravidin-HRP conjugate (Sigma) was diluted 1:1000 in PBS-Tx. Stained testes were washed in PBS then mounted in 85% glycerol, and examined with Olympus SZX 12 and BX 50 microscopes.

**RESULTS**

**aly encodes a male germline-dependent transcript expressed in early primary spermatocytes**

We cloned aly by a combination of fine structure recombination mapping, deletion analysis and mapping of RFLPs associated with insertion alleles. The aly mutation mapped 0.13±0.25 mu distal to P[w⁺] BGT-SO19 inserted in 63A4-6 (Fig. 1A). DNA flanking the P[w⁺] BGT-SO19 insertion was used to start a bacteriophage λ genomic walk.

Complementation of aly by a 1.85 kb germline-dependent transcript in males. A probe encompassing 18 kb of genomic sequence from −24 kb to −42 kb of the walk detected a single 1.85 kb transcript in northern blots of poly-A⁺-selected RNA from wild-type males (not shown, but see Fig. 2A) but not from germline-less males (not shown). A 1.5 kb cDNA obtained by screening a testis library with the same probe (Materials and Methods) also recognised the 1.85 kb transcript in poly-A⁺ RNA from wild-type males. This transcript was detected in RNA from males homozygous for aly1, a temperature-sensitive allele, but not in males homozygous for the hybrid-dysgenesis induced alleles aly4, aly5 or aly6 (Fig. 2A). The 1.85 kb transcript is likely to be a product of the aly locus rather than a downstream transcriptional target of the meiotic arrest gene pathway since it was detected in northern blots of poly-A⁺ RNA from males homozygous for can4, m10 (Fig. 2A) and sa1 (data not shown). A much less abundant transcript at 1.5 kb was also detected in the more heavily loaded lanes using the cDNA probe.

Compared to the sequences of the 1.5 kb cDNA, a 5' RACE product derived from testis RNA (See Materials and Methods) and genomic clones from the Aly region indicated that the 1.85 kb transcription unit had two small introns (lower case in Fig. 1C). The first intron was in the 5' UTR, the second 92 codons into the predicted protein. Conceptual translation revealed an ORF encoding a predicted protein of 534 amino acids, 62 kDa (Fig. 1C).

Several of the aly mutant alleles had sequence alterations that alter the predicted protein, confirming that the transcription unit identified from the aly region was indeed the aly gene (Fig. 1C). In addition to the eight published alleles (Lin et al., 1996), four new EMS-induced aly alleles (aly53-4302, aly53-4307, aly53-1393 and aly53-3504) that failed to complement aly5 and each other, were generated in a large scale mutagenesis screen (B. Wakimoto and D. Lindsley, unpublished observations). The P-element hybrid dysgenesis-induced allele aly5 had an insertion of the transposon Hobo associated with a 10 bp duplication of the genomic DNA within the predicted open reading frame (Fig. 1C, after base 1188). The EMS induced alleles aly2 and aly53-3504 had nonsense mutations that would truncate the predicted Aly protein at amino acid positions 414 and 189, respectively. In both aly53-4302 and aly53-4307 the final eight bases of the second intron were replaced with a different sequence of six bases, deleting the splice acceptor site. If this lesion resulted in failure to splice out the intron, the resulting mRNA would encode a truncated protein containing 92 amino acids of the normal protein, followed by a novel sequence of 36 amino acids. The EMS induced aly53-1393 allele had a missense mutation such that Val150 was changed to glutamic acid. The four new EMS alleles were induced on a different background chromosome from the previously described alleles. They shared several
aly is a member of a conserved gene family that includes the *C. elegans* negative regulator of vulval induction, lin-9

BLAST searches of sequence databases identified *aly* as one of two *Drosophila* homologues of the *C. elegans* gene lin-9 (Beitel et al., 2000; Fig. 3). The other homologue (86E4.4) had been identified by the *European Drosophila* genome project. Sequences with significant homology to this family of proteins were also identified from the *Arabidopsis thaliana* genomic sequence and the other alleles. These compared with the previously determined silent polymorphisms and four polymorphisms (804, A→T and 4745, C→T). The affected amino acids in these alleles are circled. Single underlined amino acids represent predicted nuclear localisation signals.

![Fig. 1. Positional cloning of *aly*. (A) Genomic walk in the *aly* region, proximal towards the right, distal towards the left. 0 kb is the position of the *P(*w*)* element insertion. (Df *aly*) a 2 kb viable and fertile deletion derived from BGT-SO19. Inverted triangles represent insertions detected by Southern blotting in the *aly* alleles produced by *P*-element hybrid dysgenesis. (B) A germ-line-dependent transcript was detected by northern blotting analysis of whole male polyA*+*-selected RNA with a 24 to 42 probe. Inverted triangles represent the position of the *aly* insertions relative to this transcription unit. B, *BamH1*; E, EcoRI; S, SalI. (C) Genomic DNA sequence of *aly* from the 5' end of the longest RACE product, to the start of the poly-A tail, with the conceptual translation to * Aly* protein shown below. Introns are shown in lower case. Boxed regions represent the mutations in *aly*<sup>c.3-360</sup> and *aly*<sup>c.3-430</sup> (504-111, ttcttag→ttggt) and *aly*<sup>5</sup> (1179-1188, duplication and hobo insertion). Underlined bases represent mutations in *aly*<sup>c.3-360</sup> (685, T→A), *aly*<sup>c.3-1393</sup> (804, A→T) and *aly*<sup>2</sup> (1475, C→T). The affected amino acids in these alleles are circled. Single underlined amino acids represent predicted nuclear localisation signals.
projects. Multiple sequence (Clustal W) alignments revealed two distinct domains of homology (Fig. 3; Table 1). For simplicity only one plant sequence is shown in the alignments. On average, pairwise comparisons (Clustal W) within Region 1 showed 32% amino acid identity, 53% similarity. The second homology domain (Region 2) was less well conserved, especially in the Arabidopsis sequence, where a putative divergent Region 2 was identified (hatched box in Fig. 3A). On average, pairwise comparisons within Region 2 showed 22% aa identity, 42% similarity, excluding those between the very well conserved vertebrate proteins, and the divergent Region 2 from Arabidopsis. The spacing between Regions 1 and 2 varied somewhat between the homologues. No significant similarities between the proteins in pairwise comparisons were detected outside the two conserved domains, nor were any similarities detected to any other proteins in the sequence databases. The second Drosophila homologue has a long C-terminal region that includes a leucine zipper motif. The Arabidopsis homologue appears to have an extended N-terminal region. However, as the Arabidopsis protein is based on GGRAIL and GenScan predictions on genomic sequence, rather than on cDNA, it is not clear if these predicted exons are actually present in the mature transcript. All the database entries for the vertebrate sequences were derived from single sequencing runs on cDNAs, so the sequences of the entire transcription units are not available. However, given that both homology regions are present in the zebrafish predicted protein, we expect to find the first homology region in the mouse and human homologues when full-length cDNA sequences become available.

A strikingly conserved feature of all the homologues where sequence of the first conserved region was available was the presence of a nuclear localisation signal (NLS) predicted by PSORT within this region (http://psort.nibb.ac.jp:8800/) (underlined in Fig. 1C, asterisks in Fig. 3A,B). In aly, this fits the consensus for a bipartite NLS: two basic residues, ten residue spacer, and another basic region consisting of at least three out of five basic residues (Robbins et al., 1991). The predicted NLS of the other homologues fits the classic consensus of four residues, at least three basic, the other any of K, R, P or H. The missense allele aly3-1393 changes Val150 to glutamic acid within region 1 of aly. This residue is conserved as an aliphatic amino acid (I, L or V). V150 of aly falls within the ten amino acid spacer region of the bipartite NLS.

### Table 1. Protein homology (% identity/% similarity) between Aly and homologues from other species

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Fig. 2. aly is expressed in primary spermatocytes. (A) Northern blot of poly-A+ selected RNA from whole wild-type or mutant males probed with an aly cDNA (bands at 1.85 and 1.5 kb), and a genomic probe from the can region (band at 3.2 kb and 3.0 kb in can1) as a loading control. (B) RNA in situ hybridisation of aly cDNA to a wild-type testis showing aly mRNA (dark blue/purple stain) in early primary spermatocytes (arrow), and not in the mitotic proliferation region.
**Aly is nuclear and concentrated on DNA in growing primary spermatocytes**

The *lin-9* gene of *C. elegans* has been identified as part of a genetic pathway that antagonises the EGFR-Ras-MAPK-based induction of vulval cell fate during larval development. The *lin-9* (SynMuVB) pathway is postulated to involve signalling between the hypoendrims and the vulcal precursor cells, based on mosaic analysis with different genes in the pathway (Hedgecock and Herman, 1995). Many components of the pathway have now been shown to be nuclear; some ubiquitously expressed, e.g. TAM-1 and LIN-53 (Hisie et al., 1999; Lu and Horvitz, 1998), others restricted to the vulcal precursor cells and not hypoendrims, e.g. LIN-36 (Thomas and Horvitz, 1999), and still others restricted to the hypoendrims and not vulcal precursor cells, e.g. LIN-13 (Melendez and Greenwald, 2000). The location in the cell at which LIN-9 acts, or even in which cell the protein is required is still unknown. How the *lin-9* pathway interacts with the EGFR-Ras-MAPK pathway also remains unknown. The *lin-9* pathway may act directly by inhibiting one of the components of the EGFR-Ras-MAPK cascade, or in parallel, perhaps controlling transcription of downstream targets of MAPK activation. To explore how *aly*, and by analogy *lin-9* and other *aly* homologues, may act, we examined the subcellular distribution of Aly protein.

Indirect immunofluorescence using an antibody raised against a bacterially expressed 6His-Aly fusion protein fragment (see Materials and Methods) revealed that Aly protein was nuclear, and mostly localised on the chromatin of primary spermatocytes (Fig. 4A-C, arrowhead), consistent with the predicted nuclear localisation signals in the protein sequence. The protein was not detected in the nuclei of the somatic cyst cells that surround each cyst of 16 germ cells (Fig. 4A-C, arrow). This result was expected from northern blot analysis, that indicated the *aly* transcript was germline dependent. The specificity of the antibody was confirmed by the absence of staining in spermatocyte cysts in testes from the knockout allele *aly*° (Fig. 4D-F).

The subcellular localisation of Aly protein depended on the stage of spermatocyte growth. Staining of wild-type testes by whole-mount immunohistochemistry revealed that in very early primary spermatocytes Aly protein was both nuclear and cytoplasmic (Fig. 5A,E, arrow). When the cysts started to mature, Aly protein became predominantly nuclear and was concentrated on chromatin (Fig. 5A,E, large arrowhead) as seen by immunofluorescence staining of cells at this stage of spermatocyte growth. Staining of wild-type testes by whole-mount immunohistochemistry revealed that in very early primary spermatocytes Aly protein was both nuclear and cytoplasmic (Fig. 5A,E, arrow). When the cysts started to mature, Aly protein became predominantly nuclear and was concentrated on chromatin (Fig. 5A,E, large arrowhead) as seen by immunofluorescence staining of cells at this stage of spermatocyte growth.

![Fig. 3. *aly* is a member of a conserved gene family including the *C. elegans* gene *lin-9.*](image)

(A) Diagram of the protein domain structures in the *aly* gene family; the first homology domain in dark grey, the second in light grey. Asterisks indicate the approximate positions of predicted nuclear localisation signals; the open box indicates a predicted leucine zipper motif in 86e4.4; the hatched region indicates *Arabidopsis* sequence with weak homology to the second conserved domain from the other proteins, but not included in the protein alignments.

(B,C) Alignment of the first (B) and second (C) regions of homology in the gene family. Black boxes indicate residues identical in 3/5 or more of the sequences; grey boxes indicate residues similar in 3/5 or more sequences; asterisks indicate predicted NLS. Sequence Accession Numbers: *aly*, AJ277307; 86E4.4, AL021086; *lin-9*, Z11115; *Arabidopsis*, AC007478; *Schistosoma*, A976519; *Zebrasfish*, A158690 and AW155830; mouse, AJ390179; human, AA256253.
always early is a lin-9 homologue

Fig. 4. Aly protein is nuclear in primary spermatocytes. Indirect immunofluorescence of wild type (A-C) and aly5 (D-F) testes stained for DNA (A,D) with propidium iodide and with an anti-Aly antibody (B,E). No Aly staining was detected in aly5 mutant testes. (C,F) Merged images: DNA (red), Aly (green), co-localisation (yellow). Arrowheads indicate the position of the same spermatocyte nucleus in each panel of a row; arrows indicate the position of the nucleus of one of the two somatic cyst cells associated with each cyst of primary spermatocytes, note that Aly protein was not detected in the cyst cell nuclei.

developmental stage in squashed preparations (Fig. 4). Aly protein staining became weaker as the primary spermatocytes matured and was undetectable in cells undergoing the meiotic divisions and all stages thereafter. Aly protein was not detected at the very apical tip of the testis where spermatogonia are undergoing mitotic amplification divisions, as expected from the lack of aly mRNA in those cells (Fig. 5A,E, small arrowhead). The shift of Aly protein from cytoplasm to nucleus in early primary spermatocytes in wild-type testes suggested that the nuclear localisation of the protein may be regulated, perhaps in response to a signalling event. In testes from the loss-of-function allele alyz3-1393, the defective Aly protein was exclusively cytoplasmic, both in early and later primary spermatocytes (Fig. 6B,F), supporting the possibility that the cytoplasmic form of Aly is inactive.

The Aly protein migrated as a 68 kDa doublet in polyacrylamide gels. Western blot analysis showed that the 68 kDa doublet was present in wild-type, and in can, mia and sa mutant testes, but not in the null alleles aly2, aly5, aly3-4307, aly3-3504 (Fig. 6). The Aly doublet was detected in testes from alyz3-1393 homozygotes. The upper band of the doublet was consistently weaker than the faster migrating band, but the relative abundance of the two isoforms was somewhat variable. The slower migrating isof orm was relatively more abundant in testes that contained arrested cells, suggesting that there may be a stage dependent modification of the protein. A second band was sometimes detected at about 90 kDa (WT lanes in Fig 6A); this band does not represent the Aly protein as it was not consistently present in wild-type samples (see Fig 6B), and was also occasionally detected in transcript null allele aly5 (not shown). Additionally this band was not detected by serum from the second rabbit immunised with the same fusion protein.

The aly1 allele is somewhat temperature sensitive: whereas most cysts arrest as primary spermatocytes, a few cysts complete the meiotic divisions and proceed through some differentiation in homozygous males raised at 18°C. In contrast, homozygous males raised at 25°C show a spermatocyte arrest phenotype indistinguishable from that of the null alleles (Lin et al., 1996). The 68 kDa Aly doublet was present in testes from aly1 homozygotes raised at 18°C, but not in testes extracts from aly1 males raised at 25°C. Consistent with this western blotting result, no staining above background levels was detected by whole-mount immunohistochemistry in testes of aly1 males reared at 25°C (Fig. 5D), whereas strong staining was observed in testes of

Fig. 5. Translocation of Aly protein to the nucleus correlates with Aly function. Whole mount immunohistochemistry of wild type and mutant testes stained with the anti-Aly antibody. (A,E) In wild type, Aly protein was absent from the apical tip (small arrowhead), present at high levels in early primary spermatocytes in both the nucleus and the cytoplasm (arrow) and localised to the nucleus of maturing primary spermatocytes (large arrowhead). (B,F) Mutant Aly protein in alyz3-1393 testes was exclusively cytoplasmic. Aly protein was nuclear in primary spermatocytes of aly1 homozygotes raised at 18°C (C,G) but absent from testes dissected from aly1 homozygotes raised at 25°C (D). Arrowhead in C indicates a cyst of cells which had progressed through the meiotic divisions, and which showed no Aly immunoreactivity. (H) Aly protein was present and nuclear in spermatocytes from can1 homozygotes.
**Fig. 6.** Aly protein expression in wild type and mutant testes. (Left) Aly protein runs as a 68 kDa doublet, in extracts from wild-type testes. Normal levels of protein were detected in the testes of aly1 males grown at 18°C. Some protein was also detected in alyz3-1393 mutant testes. No protein was detected in aly1 or from aly5, alyz3-3504 and alyz3-3504 mutant testes. The band at 90 kDa in some lanes is due to nonspecific crossreaction and was not always detected. (Right) Normal, or slightly elevated levels, of Aly protein were detected in can, mia and sa mutant testes.

**Fig. 7.** Phosphorylation of MAPK occurs normally in aly mutant testes. Testis extracts from wild type, aly5, alyz3-1393, and can1 mutant males were probed with the monoclonal antibody dp-ERK (MAPK-YT), which recognises only the di-phosphorylated (dp), and therefore activated, form of the MAPK protein ERK (top panel). They were washed and reprobed with the antibody a-ERK1/ERK2, which recognises both isoforms of the protein (bottom panel).

**aly** males reared at 18°C (Fig. 5C,G). In testes from aly males reared at 18°C, males, as in wild type, Aly protein was first detected throughout the nucleus and cytoplasm in early primary spermatocytes. In slightly older spermatocytes, the aly protein showed strong localisation on the chromatin, where it persisted in the arrested cells that accumulated at 18°C. Aly protein was not detected in the occasional cysts where meiotic divisions had occurred (arrow in Fig. 5C). The localisation of Aly protein in can1 mutant testes (Fig. 5H) and in mia and sa2 (not shown) was essentially the same as in aly reared at 18°C, except that no meiotic or post-meiotic cysts were present.

**MAPK is activated in both wild-type and aly mutant testes**

The *C. elegans* homologue of aly, lin-9, antagonises induction of vulval differentiation by the EGFR/Ras/MAPK pathway. To test whether *aly* might act to directly downregulate Ras pathway signalling in the *Drosophila* testis, we examined the phosphorylation (and thus the activation) state of MAP kinase in wild-type and aly mutant testes. *Drosophila* has a single homologue of the ERK MAP kinase, encoded by the *rolled* gene. The di-phosphorylated active form of this 44 kDa protein is recognised by the dp-ERK antibody raised against the phosphorylated activation loop of vertebrate ERK1 and ERK2 (Gabay et al., 1997). No differences in the overall level or phosphorylation state of ERK/Rolled protein were detected by western blot analysis of testis extracts from aly5 or alyz3-1393 mutant males compared with wild type (Fig. 7). This suggests that *aly*, and by analogy *lin-9*, acts downstream of MAP kinase activation, or in a separate pathway, to control target gene transcription.

**DISCUSSION**

The *aly* gene and its homologues act at the intersection of tumour suppressor, cell cycle control and terminal differentiation pathways. The Aly protein of *Drosophila* regulates both male meiotic cell cycle progression and the terminal differentiation programme of spermiogenesis by activating the transcription of genes required for both processes. Germ cells in aly mutant testes fail to progress beyond the mature primary spermatocyte stage, owing to lack of both key cell cycle transcripts required to enter the meiotic divisions, and of transcripts for proteins involved in the morphological changes of spermatid differentiation. *aly* is expressed in primary spermatocytes, the cells that show defects in aly mutants, suggesting a cell autonomous function. Aly protein is localised to the nucleus of maturing primary spermatocytes, where it appears to be associated with chromatin. Mutations in aly cause defects in the appearance of primary spermatocyte chromosomes, consistent with a role for *aly* in chromatin structure (Lin et al., 1996).

The *C. elegans* homologue of aly, lin-9, acts in a pathway with the Rb tumour suppressor protein LIN-35 to antagonise RTK-Ras-MAPK signalling during vulval development, influencing the choice of terminal differentiation pathway. Vulval formation in *C. elegans* is controlled via an inductive signalling pathway in which the anchor cell of the gonad signals to the overlying ventral ectodermal cells of the vulval equivalence group, P3.p-P8.p (reviewed in Kornfeld, 1997). This signal activates an RTK-Ras-MAPK signal transduction pathway in P6.p, causing this cell to adopt a primary vulval cell fate, and to induce its neighbours P5.p and P7.p to adopt a secondary vulval fate. The remaining cells in the equivalence group (P3.p, P4.p and P8.p) do not adopt a vulval cell fate, instead they become hypodermal. The inductive pathway is
antagonised by the SynMuv genes, which fall into two groups that represent two genetically redundant pathways. In animals doubly homozygous mutant for any one of the five SynMuvA genes and any one of the 12 SynMuvB genes, including lin-9, all the cells in the vulval equivalence group adopt an induced cell fate (Ferguson and Horvitz, 1989; Kornfeld, 1997). The SynMuvB pathway has been proposed to repress expression of vulval genes via a complex of LIN-35 (an Rb homologue), LIN-53 (an Rb associated protein) and histone deacetylase, with the RTK-Ras-MAPK signal relieving this repression to activate vulval gene transcription (Lu and Horvitz, 1998). Ras pathway signalling has been shown to result directly in inactivation of Rb after mitogen stimulation in proliferating mammalian tissue culture cells via the interaction of Rb with Raf1 (Wang et al., 1998).

Our results show that activation of MAP kinase in the testis is not dependent upon the activity of the lin-9 homologue aly. If aly antagonised a MAPK signalling pathway by preventing the phosphorylation and activation of ERK we would expect that the mutant testes would have excess di-phosphorylated, active, ERK when compared with wild type. We were unable to detect differences in the level of total or active ERK between wild-type and mutant testes, indicating that aly acts at the level of downstream effectors, or in a parallel pathway. This is consistent with the model proposed by Lu and Horvitz (1998) that the role of the SynMuv B genes is to maintain a repressor complex at the promoters of vulval differentiation genes. The activation of MAP kinase in P6.p in response to the anchor cell signal would then lead to relief of this repression and transcription of target genes.

Genetic mosaic analysis of mutations in the SynMuvB pathway suggested that some members act in the hypodermis (lin-15B, lin-37) (Clark et al., 1994; Hedgecock and Herman, 1995; Huang et al., 1994), while others function in the vulval precursor cells (lin-35, lin-36, lin-53) (Lu and Horvitz, 1998; Thomas and Horvitz, 1999). The SynMuvB pathway was therefore proposed to comprise an intercellular signalling pathway from the hypodermis to the vulval precursor cells (reviewed in Kornfeld, 1997). The lineage requirement for lin-9 function in C. elegans has not been tested. The cell autonomous activity of aly suggests that lin-9 will also have a cell autonomous role. In the nucleus, Aly protein could interact with homologues of other cell autonomous, nuclear, components of the SynMuvB pathway. Drosophila homologues of lin-35 (RbF), lin-53 (p55 subunit of chromatin assembly factor) and hda (histone deacetylase) have been described, although no Drosophila homologue of lin-36 has yet been identified.

How might Aly control transcription?

Wild-type function of the aly gene is required directly or indirectly for transcription of specific G2-M cell cycle and terminal differentiation genes in primary spermatocytes (White-Cooper et al., 1998), whereas the SynMuvB pathway is usually thought of as repressing activation of transcription of vulval genes in P5.p, P6.p and P7.p. It is also possible that the SynMuvB proteins help promote hypodermal cell fate by directly increasing transcription of genes required for hypodermal differentiation in P3.p, P4.p and P8.p. The predicted Aly protein contains neither a predicted DNA binding domain nor any domain that matches known transcriptional activators, yet it is required for the transcriptional activation of many target genes in primary spermatocytes. The Aly protein could act as a transcriptional co-activator; a physical interaction between Aly and one or more transcription factors could be responsible for the observed localisation of Aly protein to chromatin. Drosophila E2F2, which is transcribed in the testis in primary spermatocytes in a pattern very similar to that of aly (H. W.-C., unpublished observations), is a candidate aly regulated or associated transcription factor since one role of Rb is to bind to and regulate the transcription factor E2F (reviewed in Dyson, 1998). Drosophila E2F1 promotes S-phase in embryos and induces PCNA expression in tissue culture cells (Dyson et al., 1994; Ohtani and Nevins, 1994, Duronio et al., 1995); under the same conditions Drosophila E2F2 inhibits PCNA expression in tissue culture cells (Sawado et al., 1998). Likewise, although mammalian E2F-1 induces cell cycle progression in tissue culture, E2F-1 null mice are viable but develop tumours, indicating a function in cell cycle repression. Mutant E2F-1 male mice also showed significant testicular atrophy (Yamasaki et al., 1996). Many transcriptional repressors can also act as activators depending on chromatin context, which may be modulated by the SynMuvB genes. For example the YY1 transcription factor can repress transcription from the adenoviral P5 promoter in the absence of E1A, but behaves as a co-activator in the presence of E1A. Transcriptional repression by YY1 is mediated by interaction with histone deacetylase (Shi et al., 1991; Yang et al., 1996).

Mutations in several SynMuvB genes dramatically reduce expression of transgenes in repetitive extrachromosomal arrays in C. elegans, without affecting expression of the endogenous genes or transgenes in non-repetitive arrays (Hsieh et al., 1999). Additionally several SynMuv pathway genes encode components of the NURD nucleosomal remodelling and histone deacetylase complex (Solari and Ahringer, 2000). These results suggest that one function of the SynMuvB genes is to activate transcription of genes contained within specialised chromatin architectures. Although de-acetylation of histones is often thought of in the context of transcriptional repression (reviewed in Struhl, 1998), the yeast histone deacetylase RPD3, a homologue of the NURD complex histone deacetylase HDAC1, was originally identified as a factor that is required to achieve maximal levels of both transcriptional repression and activation (Vidal and Gaber, 1991). The Aly protein of Drosophila may recruit or regulate a NURD-like complex on the bivalents in primary spermatocytes. Action of this complex could have a dual effect, reducing expression of genes not part of the terminal differentiation programme, while allowing transcription of spermatogenic genes in a specialised chromatin domain. The proposed aly modulated specific chromatin domain could then be a target for a downstream transcription factor. The observation that wild-type function of aly is required for the normal appearance of chromatin in primary spermatocytes (Lin et al., 1996) is consistent with this proposed role for aly in chromatin structure.

Translocation of Aly protein from the cytoplasm to the nucleus may represent an important control point. The protein encoded by the aly<sup>z3-1393</sup> allele fails to enter the nucleus, despite the presence of two consensus predicted nuclear localisation signals. Failure of aly<sup>z3-1393</sup> mutant protein to
enter the nucleus could be explained if translocation to the nucleus is inhibited by phosphorylation of Aly protein, in a manner similar to that observed for the cell cycle-regulated nuclear localisation of the yeast SWI5 transcription factor (Moll et al., 1991). Like aly SWIS contains a bipartite NLS. Phosphorylation of three serine residues close to this NLS prevents the nuclear accumulation of the SWIS protein. S161 of Aly protein, two residues from the second basic domain of the bipartite NLS, is a good match to the consensus for cAMP-dependent protein kinase. This serine was conserved in all the aly homologues identified, where it lies two residues away from a classical NLS. The defective aly<sup>53-192</sup> protein has an acidic residue close to the NLS, which may allow it to adopt a conformation mimicking that of the phosphorylated form.

The *C. elegans* SynMuvA and SynMuvB pathways are genetically redundant in vulval development, but not in all tissues. Similarly, although we have only detected defects in the male germline in aly mutant flies (Lin et al., 1996), aly may function at other stages of development if a genetically redundant pathway is active. This remains a possibility as a function at other stages of development if a genetically redundant in vulval development, but not in all tissues. Alternatively, we have only detected defects in the male germline in aly mutant flies (Lin et al., 1996), aly may function at other stages of development if a genetically redundant pathway is active. This remains a possibility as a low level of aly message was detected in adult females by RT-PCR (H. W.-C. unpublished observations). Alternatively, the second *Drosophila* lin-9 homologue, 86E4.4, could carry out the lin-9-like function at earlier stages of *Drosophila* development. The conservation of aly in many phyla suggests a SynMuvB-like pathway may be a conserved feature in many different organisms, ranging from plants to vertebrates. It will be interesting to determine whether the aly family functions in mammals to coordinate meiotic divisions with gamete production. Transcription of boule lies downstream of aly function (White-Cooper et al., 1998). If the mechanisms are conserved, we may expect that transcription of the boule homologues Daz and Dazl (Eberhart et al., 1996; Ruggiu et al., 1997) in mammalian spermatogenesis are dependant on aly homologues. The recent discovery in plants of both Rb proteins and other components of the Rb pathway (reviewed in de Jager and Murray, 1999) supports the hypothesis that a SynMuvB pathway may have a conserved role in allowing multicellular organisms to evolve complex structures consisting of many different cell types, whose normal development depends on the coupling of cell cycle controls with cellular differentiation.

The mechanism by which SynMuv genes control the choice of differentiation pathway is still very poorly understood. The SynMuv genes, including *Rb* and *aly*, may not always be involved in negative regulation of RTK-RAS-MAP kinase signalling. Rather, SynMuv pathway genes and *aly* could play a more general role in regulating differentiation, both repressing transcription of certain genes and being required for activation of others in a specialised chromatin context. In the case of the *C. elegans* vulval precursor cells, *Rb* and the SynMuv genes could counteract EGFR pathway signalling by affecting chromatin in the region of EGFR-RAS-MAP kinase target genes. In *Drosophila* primary spermatocytes, *aly* and its partners could also affect expression of meiotic cell cycle and terminal differentiation genes via effects on chromatin.

We thank Greg Beitel, Stuart Kim, Min Han, Luke Alphay and Myles Axton for critical reading of the manuscript, and members of the Fuller, Alphay, Axton and White-Cooper laboratories for helpful discussions throughout this work. We thank Drew Suem for help with the recombination mapping of *aly*. We are also indebted to Greg Beitel for communication of unpublished data, and Barbara Wakimoto, Dan Lindsay and Charles Zuker for the new *aly* alleles. This work was supported by an NIH grant (1R01 HD32936) to M. T., a Walter and Idun Berry Fellowship, an MRC grant (#G9811588) and Royal Society research grant (#20010) to H. W.-C.

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