

Painting of fourth in genus *Drosophila* suggests autosome-specific gene regulation

Jan Larsson*, Malin J. Svensson, Per Stenberg, and Maria Mäkitalo

Umeå Centrum för Molekylär Patogenes, Umeå University, SE-901 87 Umeå, Sweden

Edited by Mary-Lou Pardue, Massachusetts Institute of Technology, Cambridge, MA, and approved May 17, 2004 (received for review February 11, 2004)

Painting of fourth (POF) is a chromosome-specific protein in *Drosophila* and represents the first example of an autosome-specific protein. POF binds to chromosome 4 in *Drosophila melanogaster*, initiating at the proximal region, followed by a spreading dependent on chromosome 4-specific sequences or structures. Chromosome-specific gene regulation is known thus far only as a mechanism to equalize the transcriptional activity of the single male X chromosome with that of the two female X chromosomes. In *Drosophila*, a complex including the male-specific lethal proteins, "paints" the male X chromosome, mediating its hypertranscription, explained to some extent by the acetylation of lysine 16 on histone H4. Here, we show that *Pof* is essential for viability in both sexes and for female fertility. POF binding to an autosome, the F element, is conserved in genus *Drosophila*, indicating functional conservation of the autosome specificity. In three of nine studied species, POF binds to the male X chromosome. When bound to the male X, it also colocalizes with the dosage compensation protein male-specific lethal 3, suggesting a relationship to dosage compensation. The chromosome specificity is determined at the species level and not by the amino acid sequence. We argue that POF is involved in a chromosome-specific regulatory function.

Chromosome-specific gene regulation has so far been identified only as the mechanism for dosage compensation of sex chromosomes. Dosage compensation equalizes the transcriptional activities of the two X chromosomes in the homogametic sex with that of the single X chromosome in the heterogametic sex (1, 2). Dosage compensation strategies vary widely between species (3); in female mammals, one X chromosome is inactivated and forms the Barr body, whereas in *Drosophila melanogaster*, dosage compensation is achieved by increasing transcription of the single male X chromosome 2-fold (1, 3).

In *D. melanogaster*, two noncoding RNAs, *roX1* and *roX2*, are essential components of the dosage compensation system (4). These noncoding RNAs, together with the five MSL (male-specific lethal) proteins, "paint" the dosage-compensated male X chromosome (5). The MSL complex mediates acetylation of H4 at lysine 16 on the male X chromosome (6), which in part explains the subsequent hypertranscription. The role played by the *roX* RNAs in *Drosophila* has suggested parallels between the fly and the mammalian dosage-compensating systems. In female mammals, a large noncoding RNA, the product of the *Xist* gene, plays a critical role both in the choice of which X chromosome remains active and in the initial spread and establishment of silencing on the inactivated X chromosome (7). A connection between BRCA1, a breast and ovarian tumor suppressor, and *Xist* RNA has been reported (8). BRCA1 associates with *Xist* RNA and supports X inactivation, and is thus the first identified protein factor in the mammalian dosage-compensating system. A Polycomb group protein, Eed, has been shown to play a role in imprinted X inactivation; i.e., in the extra embryonic tissues (9), and is localized on the inactive X chromosome (10).

The mammalian *Xist* RNA, like the *Drosophila roX1* and *roX2* RNAs, paints the dosage-compensated X chromosome. Although the way compensation for chromosome dosage is achieved differs between mammals and *Drosophila*, the basic features of the process display striking similarities. In both

systems, chromatin complexes containing spliced, polyadenylated, noncoding RNAs become associated with the compensated chromosome and paint it by spreading along the chromosome. The painting initiates at "nucleation" site(s) and spreads from them to involve most of the chromosome. Our discovery of Painting of fourth (POF) (11), provides the first example of a chromosome-specific protein for an autosome. Unlike BRCA1, Eed, and the MSL proteins, POF paints an autosome, the fourth chromosome of *D. melanogaster*. Chromosome translocation analysis showed that the binding depends on an initiation site in the proximal region of chromosome 4 and spreads in cis to involve the entire chromosome. The spreading depends on sequences or structures specific to chromosome 4 and cannot extend to parts of other chromosomes translocated to the fourth. Spreading can also occur in trans to a paired homologue that lacks the initiation region. In the related species *Drosophila busckii*, POF paints the entire X chromosome exclusively in males, suggesting a relationship between the fourth chromosome and the X and between POF complexes and dosage compensation complexes (11). The fourth chromosome is the smallest chromosome, ≈ 5 Mb (12), and consists of a centromeric, highly condensed region that is underreplicated in polytenic tissues, and a banded, polytenized region, corresponding to cytological sections 101E–102F. Chromosome 4 is in many respects an atypical autosome. Many features indicate that the fourth chromosome of *D. melanogaster* is largely heterochromatic in nature. The heterochromatin protein HP1 is found associated with much of chromosome 4 (13), and certain histone modifications, identifying heterochromatin are enriched, e.g., H3K9-methylated (14). Repetitive elements normally confined to heterochromatin are distributed throughout the banded region of chromosome 4 (15). Furthermore, reporter genes inserted at many sites in this chromosome often display a variegated, partially repressed expression typical of heterochromatic position-effect variegation (16, 17). Chromosome 4 is also the only autosome that survives in a haploid condition (18). The majority of *Drosophila* species have a corresponding microchromosome (19) also called the F element (20). In some *Drosophila* species, such as *D. busckii*, the microchromosome appears to form the proximal part of the X chromosome (21).

Here, using genetic and molecular analysis of the *Pof* gene and deduced protein, we show that *Pof* is essential for female fertility and viability of both sexes. Immunostainings of chromosomes in different species show that the F element specificity is conserved in evolution and that POF is connected to dosage compensation. Based on this finding we conclude that the F element specificity supports a function and we speculate that this function may be

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: POF, Painting of fourth; MSL, male-specific lethal; YFP, yellow fluorescent protein; EYFP, enhanced YFP; S2, Schneider 2.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY545996).

*To whom correspondence should be addressed. E-mail: jan.larsson@ucmp.umu.se.

© 2004 by The National Academy of Sciences of the USA

a first example of chromosome-specific regulation of an auto-some.

Materials and Methods

Fly Strains and Crosses. Fly stocks from different species were provided from the Tucson and Umeå stock centers; for *D. busckii* see ref. 11. Established mutants and fly lines used in this study have been described (11, 22). Flies were cultivated in vials with potato mash-yeast-agar medium, except for *D. busckii*, which was raised in Formula 4–24 Instant medium (Carolina Biological Supply).

Generation of Pof Mutants by P Element Excision. We excised the P element inserted close to the transcription start of *Pof* in the fly line *EP (2)2285* to create short deletions in the *Pof* region. Single white-eyed, non-*Sco* males from the cross (*yw/yw; Sco/CyO* × *w¹¹¹⁸; EP (2)2285/CyO; mus309^{D3}, Δ2-3/mus309^{D2}*) were crossed to *yw; Sco/CyO* females. After 4 days, the single male was removed from the cross, DNA was prepared (23), and PCR was used to screen for short imprecise excisions. The primers used were: 5'-TCCGCATCAGAATCCTCCC-3' and 5'-GATCATCGGTGGTATCAAGG-3' to screen for promoter deletions and 5'-ATTTAACTTCGTAAGAGGGC-3' and 5'-CGACGAGTAATTTGGTACTG-3' to screen for deletions in the transcribed region. Crosses from males with excisions were continued to establish balanced stocks. The *mus309* trans-heterozygous genotype increases the yield of imprecise excisions (24).

Transgenic Flies. The *P[white⁺ Pof]* construct was prepared as follows: A genomic *Pof* fragment was amplified by using primers 5'-TATCTCGAGCTGATCGGCAAATACCCAAAATG-AAG-3' and 5'-AATCTAGAGCCATATGCAAGGAATTG-GAGAGAA-3', wild-type DNA and pfu^{TURBO} polymerase. The PCR product was cut with *XhoI/XbaI* and ligated into P{CaSpeR-4} (25). The *P[white⁺ Pof.EYFP]* construct expressing the POF complete protein with a yellow fluorescent protein (YFP) tag at the C terminus was made as follows: Primers 5'-TATCTCGAGCTGATCGGCAAATACCCAAAATG-AAG-3' and 5'-GCAGAATTCGAGGATCAGGATCGC-3' were used to amplify the *Pof* fragment. The PCR product was cut with *XhoI/EcoRI*. The enhanced YFP (EYFP)-encoding fragment was excised from the pEYFP-N1 plasmid (Clontech) by using *EcoRI/NotI*. The two fragments were purified and ligated into a *XhoI/NotI* cut P{CaSpeR-4} vector. The *P[white⁺ Drosophila ananassaePof.EYFP]* construct: The *DaPof* fragment was amplified from a *DaPof* cDNA clone by using primers 5'-GCTAGGAACTCGAGTAGGACAGTAT-3' and 5'-CGCGG-TACCGTCTCGGCATCTGATTCCAATTGAGCATAT-3' and cut with *XhoI/KpnI*. The EYFP fragment was excised from the pEYFP-N1 plasmid by using *KpnI/XbaI*. The two fragments were purified and ligated into a *XhoI/XbaI* cut pUAST vector (26). Salivary gland overexpression of DaPOF.EYFP was achieved by combining *P[white⁺ D. ananassaePof.EYFP]* with the salivary gland-specific driver *P[w⁺ GawB]1*. The constructs were sequenced to confirm the absence of mutations resulting from errors during PCR. Germ-line transformation of the construct was completed according to described methods (27), by using the *Df (1)w^{67c23}, yw*, or *w¹¹¹⁸* strain as host.

Molecular Biology. To clone the *D. ananassae Pof* cDNA a 350-bp *DaPof* fragment, isolated by PCR, ³²P-labeled by random priming was used to probe a *D. ananassae* cDNA third-instar larvae library (kindly provided by Kiyohito Yoshida, Hokkaido University, Sapporo, Japan). Inserts from two positive colonies were subcloned into pBluescript II KS(+) and sequenced. An 1,897-bp cDNA was isolated and the encoded amino acid sequence was deduced (GenBank accession no. AY545996).

RT-PCR was used to determine transcript levels in the induced *Pof* mutants. The primer pair 5'-CCACCATCTGCGACATA-AACAG-3' and 5'-CGACGAGTAATTTGGTACTG-3' will amplify only the endogenous *Pof* transcript, 5'-CCAC-ATCTGCGACATAAACAG-3' and 5'-CGTCCGCGTCCA-GCTCGACCA-3' only the *Pof.EYFP* transcript, and 5'-AAA-GACGATGCCAAGGACTCAC-3' and 5'-GCTTGCGGCC-GTGCCGACA-3' will amplify the neighboring gene *CG4806*. Poly(A)⁺ RNA was isolated as described (28). One microliter of poly(A)⁺ RNA, pd(T)_{12–18} first-strand primer, primer pairs indicated above, and Ready-to-Go RT-PCR beads (Amersham Biosciences, Piscataway, NJ) was used in each RT-PCR. The amplified products were separated on agarose gels, were visualized, and were quantified by using a Fluor-S MultiImager (Bio-Rad). Sequence alignments were performed by using BIOEDIT and CLUSTALW (29).

Antibodies. Polyclonal antibodies were raised in hen and rabbit by using a full-length bacterially expressed protein as antigen (AgriSera, Vännäs, Sweden). A construct expressing a GST-POF fusion protein was generated by cloning a *Pof* cDNA into the pGEX5X-3 vector. The fusion protein was produced and purified according to the manufacturer's instruction (Amersham Pharmacia Biosciences) by using Factor Xa cleavage to elute the untagged POF protein from a GSTrap (5 ml) column. The eluted protein was concentrated and used for immunization. The rabbit sera and the purified hen IgY were affinity-purified on an UltraLink Iodoacetyl column as described by the manufacturer (Pierce).

Immunostainings and Immunofluorescence. Polytene chromosomes were prepared and stained from the salivary glands of third-instar larvae essentially as described (11). As primary antibodies against POF a rabbit or hen polyclonal anti-POF antibody raised against a synthetic peptide (11) diluted 1:100, and rabbit or hen antibodies raised against full-length POF protein (see above, 1:400) were used. For double staining, a goat anti-MSL3 antisera, diluted 1:1,000 (kindly provided by M. Kuroda, Harvard Medical School, Boston), or rabbit anti-H4K16ac (Serotec, 1:250) were used. Anti-GFP (Living Colors Av peptide antibody, Clontech) were used to detect EYFP, diluted 1:40. As secondary antibodies, donkey anti-rabbit, donkey anti IgY and donkey anti-goat conjugated with Cy3 or FITC were used (The Jackson Laboratory), diluted 1:400. Schneider 2 (S2) cells were grown and fixed according to standard methods and were stained as described above. Live ovaries and salivary glands were dissected and mounted in halocarbon oil 27 as described (30). Preparations were analyzed by using a Zeiss Axiophot microscope equipped with a KAPPA DX30C charge-coupled device camera. Images were assembled, contrasted, and merged electronically by using PHOTOSHOP (Adobe Systems, San Jose, CA). The filter set 41028 (Chroma Technology, Rockingham, VT) was used to detect EYFP.

Results

Pof Mutants Were Isolated by P Element Excision. To learn about the *in vivo* function of *Pof* besides its binding to chromosome 4, short deletion mutants were created by imprecise excision of the EP element located at the transcription start point of *Pof* in line *EP (2)2285* (Fig. 1A). The EP insertion itself decreases the amount of transcript as seen in Fig. 1B but causes no mutant phenotype. Eight induced *Pof*-deletion alleles are shown in Fig. 1A. *Pof^{Δ4}* deletes the normal translation start point of *Pof*, but a truncated protein is formed that restores POF function (results not shown). Deletions removing the promoter region of *Pof* (*Pof^{Δ2}*; *Pof^{Δ31}*; *Pof^{Δ7}*) cause recessive lethality, which is completely rescued by a genomic copy of *Pof* carried by a transposon, i.e., *P[w⁺ Pof]* or *P[w⁺ Pof.EYFP]*. By using a sensitive RT-PCR method, we

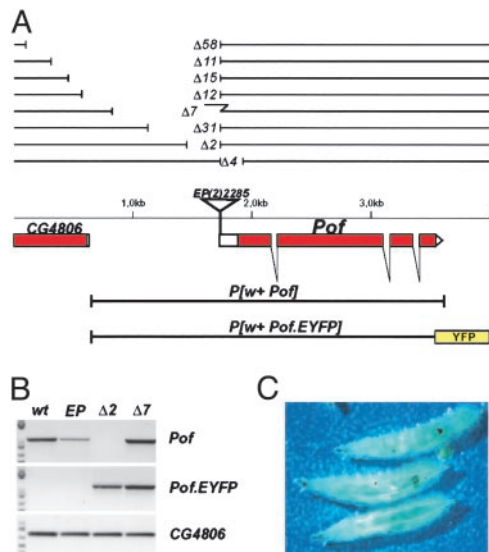


Fig. 1. Map of the *Pof* gene. (A) The exon–intron structure of the *Pof* gene is shown below the genomic DNA line. Filled boxes represent coding regions and open boxes represent untranslated sequences. The EP element insertion in the homozygous viable *EP* (2)2285 stock and the extent of induced deletions and transgenic constructs are shown. The broken line in the *Pof*^{Δ7} deletion symbolizes a 600-bp residual piece from the *P* element. The EYFP fusion sequence is indicated as a yellow box. (B) RT-PCR analysis on wild-type, *EP* (2)2285/*EP* (2)2285, *P*[w⁺ *Pof*.EYFP]; *Pof*^{Δ2}/*Pof*^{Δ2} and *P*[w⁺ *Pof*.EYFP]; *Pof*^{Δ7}/*Pof*^{Δ7} female poly(A)⁺ RNA. The primer pairs amplify only endogenously transcribed *Pof* (Top), *Pof*.EYFP (Middle), and *CG4806* (Bottom). (C) Sixteen-day-old *Pof*^{Δ2}/*Pof*^{Δ2} third-instar larvae with melanotic masses.

detect no transcript from the endogenous *Pof* in *P*[w⁺ *Pof*.EYFP]; *Pof*^{Δ2}/*Pof*^{Δ2} rescued females (Fig. 1B) nor in *P*[w⁺ *Pof*.EYFP]; *Pof*^{Δ31}/*Pof*^{Δ31} females (results not shown). The *Pof*^{Δ7} allele still expresses *Pof* RNA despite its lethality. The residual *P* element sequence seen in this allele (Fig. 1A), containing part of the *hsp70* control region, probably drives this expression, which does not restore *Pof* function. Previously (11), we have shown that only a small amount of transcript is detected in females compared to males (20- to 30-fold less). However, the high-level expression seen in males originates from the testes (results not shown). A low expression is seen in both sexes in most tissues, whereas a very high amount of transcript is found in male testes (results not shown). *Pof*^{Δ2} and *Pof*^{Δ31} abolish the low overall expression and cause lethality but do not affect the testes specific expression. Because the *P*[w⁺ *Pof*.EYFP] construct still shows correct testes expression (results not shown), we conclude that a testes-specific regulatory element is located in the 472-bp fragment between the upstream end of *P*[w⁺ *Pof*.EYFP] and the *Pof*^{Δ31} break point. The longer deletion alleles, i.e., *Pof*^{Δ58}, *Pof*^{Δ11}, *Pof*^{Δ15}, and *Pof*^{Δ12}, which also affect expression from *CG4806*, are not trans-complemented by a transgenic *Pof* construct. Homozygous *Pof*^{Δ2} or *Pof*^{Δ31} are lethal in third-instar larvae, no pupae form. By picking non-GFP homozygous first-instar larvae from a *Pof*^{Δ2}/*CyO*, *GFP* stock we could see that *Pof*^{Δ2}/*Pof*^{Δ2} hatch as first-instar larvae without significant delay. Their development is then progressively slowed down. Crawling third-instar larvae are found after 14 days, compared with 4–5 days in wild type. After 31 days, >20% of these larvae are still moving. In a majority of third-instar *Pof*^{Δ2}/*Pof*^{Δ2} larvae melanotic masses of different sizes are found (Fig. 1C). However, whether these “pseudotumours” are a direct consequence of the mutant or caused by the developmental delay is not known. As expected, no POF staining is seen on polytene chromosomes from *Pof*^{Δ2}/*Pof*^{Δ2} larvae. To investigate the role of

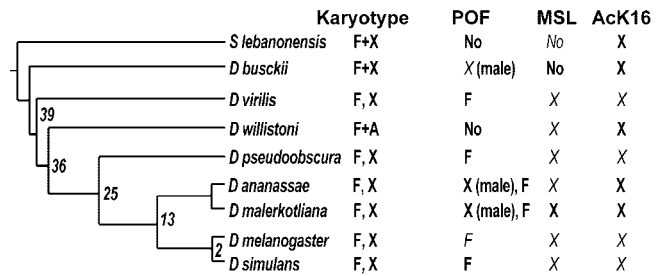


Fig. 2. Phylogenetic relationship among the species studied based on ref. 33. All species belong to the *Drosophila* genus, except *S. lebanonensis* from the *Scaptodrosophila* genus. Karyotypes are indicated, F element fused to X (F+X), F element fused to an autosome (F+A), F element fused to X (F, X), and F element fused to an autosome (F, X). POF bindings are tested in both sexes and indicated if sex-specific. The F element specificity is always seen in both sexes. No, No staining is detected. The indicated stainings for MSL and H4K16ac are specific for the male X. Results in italics have been shown (11, 31, 45).

maternal *Pof* mRNA to embryo development, we removed *Pof* in germ-line clones by using *FLP/FRT*-induced recombination. However, *Pof*^{Δ31}/*Pof*^{Δ31} germ cells arrest at an early stage of oogenesis and no eggs are produced. The effect of homozygous *Pof* mutation on male fertility has not been tested. We conclude that the *Pof* gene function is essential for female fertility and proper development in both sexes.

F Element-Specific Binding Is Conserved in Evolution. POF binds specifically to chromosome 4 in *D. melanogaster* (11) and is essential. This finding is controversial because it implies that chromosome 4 might be under a chromosome-wide control. However, it is not clear that the binding of the fourth chromosome has a function *in vivo*. To test this hypothesis, we used an evolutionary approach. We raised two new polyclonal antibodies against the whole POF protein sequence to examine different species for presence and specificity of POF. By using four different POF antibodies, we stained eight species from the genus *Drosophila* and one species from the genus *Scaptodrosophila* (*Scaptodrosophila lebanonensis*; Fig. 2). Strikingly, we find evolutionary conservation of the binding of POF to the F element. Distantly related species, such as *Drosophila virilis* and *Drosophila pseudoobscura*, still show a specific binding of POF to the F element (Fig. 3A). In *D. virilis*, POF also decorates the nucleolus. We believe that this staining is also specific because we find it in the close relative species *Drosophila americana americana* as well (results not shown). The F element-specific binding is always seen in both sexes in the tested species. The conservation through evolution suggests that POF binding to the F element confers some selective advantage *in vivo*. The binding results are summarized in Fig. 2.

Male X-Specific Binding Is Conserved in Evolution. Our earlier results showing POF staining on the male X-chromosome in *D. busckii* supported the suggested relationship between the F element and the X chromosome and suggested that POF may be part of an ancient dosage compensation system (11). No MSL binding has been found on *D. busckii* male X chromosome (ref. 31 and results not shown; MSL1, 2, 3, maleless, males absent on the first tested); however, the H4K16 acetylation is conserved and colocalizes with POF (Fig. 6, which is published as supporting information on the PNAS web site). We found that POF binds to both arms of the male X chromosome also in *D. ananassae* (Fig. 3A) as well as to the F element in both sexes. Also in the *D. ananassae* relative *Drosophila malerkotliana* a specificity to the male X chromosome is seen (Fig. 2, and Fig. 7, which is published as supporting information on the PNAS web site). Furthermore, on the X chromosome, POF perfectly colocalizes with the MSL

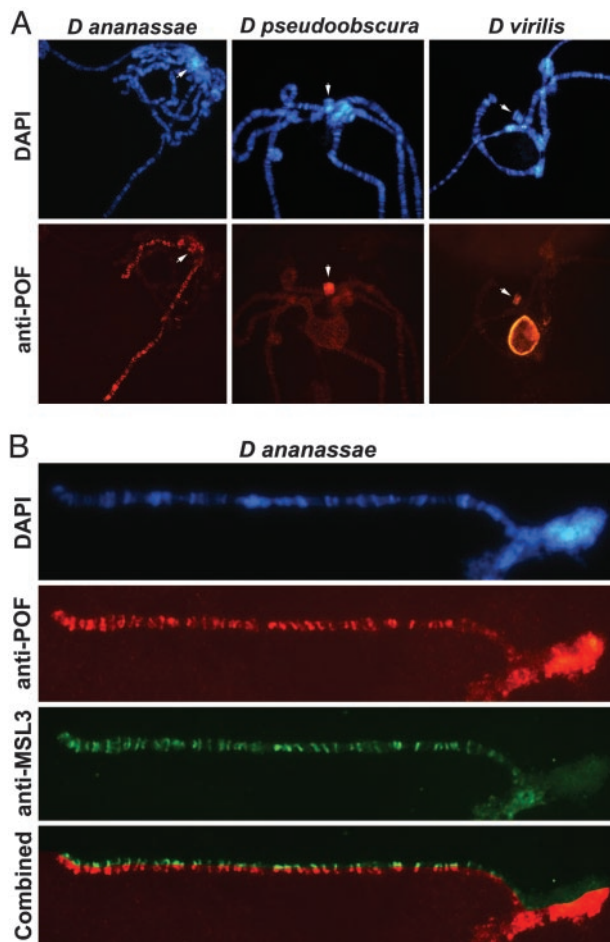


Fig. 3. Localization of POF on salivary gland chromosomes from different species. (A) POF binds to the male X chromosome and to the F element in *D. ananassae* (Left) and to the F element in *D. pseudoobscura* and *D. virilis*. In *D. virilis*, the nucleolus is also decorated by POF. An arrow indicates the F element. (B) Higher magnification of a stretched male X chromosome (right arm) and the F element in *D. ananassae*. The combined image (Bottom) shows that POF colocalizes perfectly with MSL3 on the X chromosome, whereas only POF decorates the F element.

complex, as shown by double staining with MSL3 in Figs. 3B and 7. This finding indicates that POF binds to the same chromosomal loci as the dosage-compensation complex and may be included in the complex. We did not observe binding of POF to any chromosome in two species. There was no signal in *Scaptodrosophila lebanonensis*, which is the form farthest away from *D. melanogaster*. We tested *S. lebanonensis* because it has been suggested to have the F element fused to the X chromosome in the fashion of *D. busckii* (32). We did not see any signal in *Drosophila willistoni* either. It is interesting to note that *D. willistoni* is the only tested species that has the F element fused to one of the major autosomes (32). It is also the only *Drosophila* where we fail to detect POF with any of the four used antibodies.

The Chromosome-Specific Property Is Species-Specific. We were interested to test the determinant for the chromosome specificity. We therefore cloned the *Pof* gene from *D. ananassae*, in which POF binds the male X. We fused the *DaPof* gene to EYFP, put it under control of a UAS promoter, and introduced it into *D. melanogaster*, $P[w^+ DaPof.EYFP]$. A genomic *D. melanogaster Pof* was also fused to EYFP to be used as a control of the system, $P[w^+ Pof.EYFP]$ (Fig. 1A). Salivary glands dissected from $P[w^+$

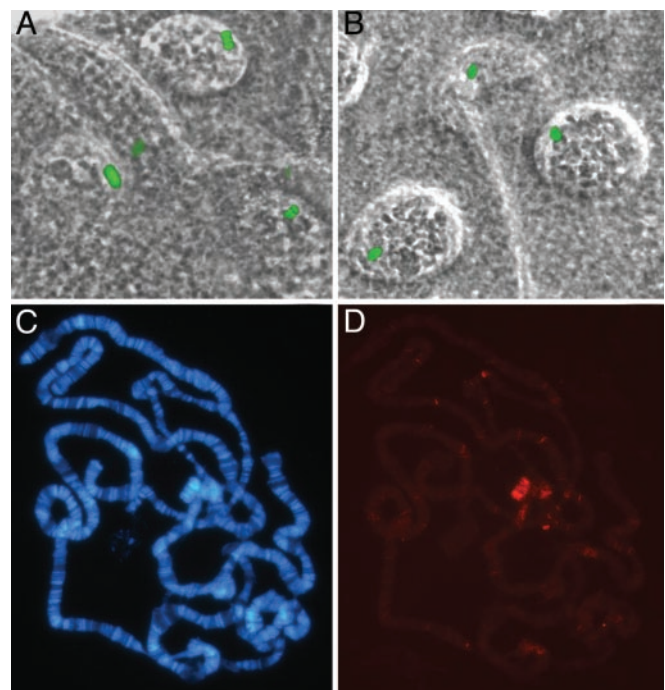


Fig. 4. Localization of DmPOF and DaPOF in *D. melanogaster* salivary glands. (A) Unfixed salivary gland showing DmPof.EYFP fluorescence of chromosome 4 in the nuclei. The phase contrast and the fluorescent images are merged. (B) Unfixed salivary gland showing DaPof.EYFP fluorescence of chromosome 4. (C and D) Localization of DaPOF.EYFP on *D. melanogaster* salivary gland chromosomes as detected by anti-EYFP antibody. DAPI (C) and anti-EYFP visualized by Cy3 (D).

Pof.EYFP] transgenic larvae show a bright fluorescence of a chromosomal part in the nuclei corresponding to the fourth chromosome (Fig. 4A). This finding tells us that POF binds the fourth chromosome specifically also *in vivo* and that the formaldehyde fixation used for preparing chromosomes does not alter the *in vivo* situation. The $P[w^+ DaPof.EYFP]$ transgenic larvae have a clearly fluorescent fourth chromosome, indicating that also the DaPOF protein binds specifically to the F element when introduced in *D. melanogaster*, seen both *in vivo* (Fig. 4B) and in formaldehyde-fixed squashes (Fig. 4C). DaPOF is found preferentially in interbands rather than in condensed chromatin, which is similar to the DmPOF. The fourth chromosome specificity is seen in three tested independent $P[w^+ DaPof.EYFP]$ lines without a GAL4 driver. We have not succeeded in rescuing *Pof* mutants by using the UAS-driven DaPOF transgene. Whether this outcome is caused by a dysfunction of the DaPOF protein or a failure to achieve proper expression is not known. When overexpressed in salivary glands, in contrast to DmPOF (11), the DaPOF protein binds to all chromosomes. The results suggest that the chromosome specificity of POF is at the species level and is not determined by the amino acid sequence *per se*. This finding is confirmed by sequence comparison where the *D. pseudoobscura* POF protein (F binding) is not more similar to the *D. melanogaster* sequence (46% identical) than to the *D. ananassae* sequence (51%) (Fig. 8, which is published as supporting information on the PNAS web site).

Chromosome 4 Specificity Is Seen in Different Cell Types. We next wanted to see whether POF binding to the fourth chromosome is unique to polytene chromosomes or polyploid tissues. We therefore examined antibody staining and POF.EYFP fluorescence in different cells and tissues. Antibody staining of S2 cells shows that POF is localized at specific foci in the nuclei (Fig. 5

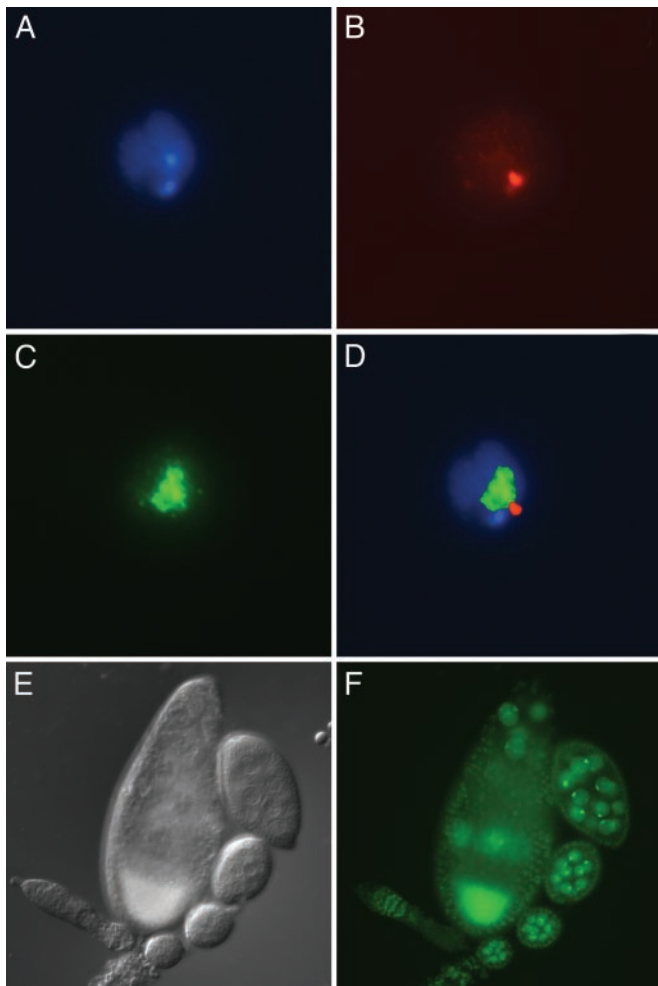


Fig. 5. POE localization in S2 cells and in ovaries. (A–D) S2 cells: DAPI (A), anti-POE (B), anti-MSL3 (C), and merged (D). The merged image shows that POE staining is separable from the MSL3-labeled X chromosome. Immunofluorescence of POE.EYFP as detected in live ovary preparations. (E) Nomarski image. (F) EYFP fluorescent image. The strong signal in the oocyte is caused by autofluorescence.

A–D). These foci are separable from the male X visualized by anti-MSL3 staining. By comparing the sizes, we suggest that POE decorates the fourth chromosome also in S2 cells. The staining is seen as one or two clear foci, suggesting that in some nuclei the two fourth chromosomes are paired or are colocalized. During mitosis, POE is not seen on the fourth chromosome, which is in contrast to MSL3 that is still detectable at the metaphase X chromosome (results not shown). By using the *P/w⁺ Pof.EYFP* transgenic line, we also tested other tissues. We detect POE, presumably on chromosome 4, in a variety of cell types, including ovary nurse cells (Fig. 5 E and F) and follicle cells (data not shown). The presence of POE in these cell types may explain the sterility of *Pof^{Δ31}/Pof^{Δ31}* germ-line clones.

Discussion

Chromosome-specific binding of proteins has so far been described only for the dosage-compensated X chromosome and the binding of POE to the entire F element is to our knowledge a unique characteristic. Although heterochromatin proteins, e.g., HP1 (13), have been shown to bind extensively to chromosome 4 and certain histone modifications are enriched, e.g., H3K9-methylated (14), this finding is accompanied by association to other heterochromatic regions, whereas POE is strictly specific

for chromosome 4 and is not detectable in the general centromeric region. By inducing deletions in the promoter region of *Pof*, presumably null mutations according to RT-PCR, we show that *Pof* is essential for oogenesis in females and viability in both sexes. The phenotypic manifestation of mutated *Pof*, i.e., prolonged larval development, melanotic masses, and failure to form pupae, is hard to interpret. At this point, we cannot distinguish the direct consequences of the *Pof* mutation from indirect effects. We conclude that *Pof* is essential, which raises the controversial question whether the F element may be under a chromosome-specific control. To link the binding of POE to the F element with a function, we undertook an evolutionary study, arguing that conservation of POE binding to the F element indicates function. Our study of different species within the genus *Drosophila* shows this conclusion to be the case. The F element is found to be decorated by POE in both *D. pseudoobscura* and *D. virilis*, species that diverged from *D. melanogaster* 25 and 39 million years ago, respectively (33), and we conclude that the F element-binding property serves a function. We have previously shown that the binding of POE in *D. melanogaster* depends on an initiation site in the proximal region of chromosome 4 and spreads in cis to involve the entire chromosome. The spreading depends on sequences or structures specific to chromosome 4 and cannot extend to parts of other chromosomes translocated to the fourth (11). In both *D. virilis* and *Drosophila simulans*, the whole banded region of chromosome 4 has been inverted relative to e.g., *D. melanogaster* (34), yet POE binds to their respective F elements. This discovery shows that the gene order of the chromosome is not involved in the spreading process.

Previously, we have shown that in *D. busckii*, POE paints the entire X chromosome exclusively in males, suggesting a relationship between the fourth chromosome and the X and between POE complexes and dosage compensation complexes (11). Although none of the MSL proteins has so far been detected on the male *D. busckii* X chromosome, by using the available antibodies, the fact that the H4K16 acetylation is conserved argues that a similar dosage compensation system is used. Our finding of POE binding to the male X chromosome in *D. ananassae* strengthens the suggested relationship between POE and dosage compensation. The colocalization of POE and MSL3 in these species further suggests this finding to be the case. When introduced into *D. melanogaster*, the DaPOE binds to the fourth chromosome and not to X, in neither male nor female. This finding indicates that the chromosome specificity of POE is determined at the species level rather than at protein sequence level, and may include interactions with other proteins or include noncoding RNAs in similarity to the dosage compensation systems in *Drosophila* and mammals (1).

Considering this association, we suggest that the function of POE binding to the F element is of regulatory character. Chromosome 4 is a unique chromosome in several aspects. One is its heterochromatic nature, including binding of the heterochromatic protein HP1 (35), and late replication of the chromosome as a whole (36). It has been suggested that heterochromatic and euchromatic regions are interspersed along the banded region of the chromosome (37). Another aspect is that similar to the X chromosome but in contrast to the autosomes, haplo-4 and triplo-4 flies are viable and fertile, although haplo-4 females breed poorly (19), suggesting a mechanism of dosage compensation (18). Alternatively, the small size of chromosome 4 may account for the haplosufficiency. In general, deletions that extend over more than one of Bridges numbered chromosomal divisions are lethal (38). However, exceptions exist as exemplified by the large deletions *Df(2L)H* and *Df(3L)Vn* (19). The numbered divisions are estimated to be in the range of 800–1,500 kb with few exceptions (39) and the *Df(2L)H* and *Df(3L)Vn* deletions span <2.8 and <1.7 Mb, respectively. Although the

banded (and sequenced) region of chromosome 4 is 1,230 kb, the chromosome as a whole is estimated to 4.5–5.2 Mb (12). This finding means that chromosome 4 is the only haplosufficient autosome and is also by far the longest region of haplosufficiency among autosomes in *D. melanogaster*. In this respect, it is interesting to note that in the only species tested within the genus *Drosophila* where POF is not detected, *D. willistoni*, the F element is fused to one of the autosomes, element E corresponding to 3R in *D. melanogaster* (32). We speculate that the potential POF-supported regulatory mechanism is not needed when the F element is part of one of the major autosomes. So far, there are to our knowledge, no proof of chromosome specific regulation on autosomes. Chromosome number differences between related species must be considered as an argument against this possibility. Extreme examples of different diploid numbers between related species exist, as exemplified by the barking deers, muntjacs (40). The extremes among these, the Indian muntjac (*Muntiacus muntjac*) with $2n = 6$ and the Chinese muntjac (*Muntiacus reevesi*) with $2n = 46$. Despite the karyotypic differences, these species are morphologically similar, and can hybridize, although the hybrids are sterile. It is argued that the correct somatic development of these hybrids implies that it is irrelevant if the genes underlying the developmental circuit are on 6 or 46 chromosomes (40). Although, in general, diploid species within a genus show a narrow range of chromosome numbers, examples of high karyological differences exist. Cytogenetic studies of titi monkeys (*Callicebus*) have provided a similar example, with diploid numbers ranging from $2n = 50$ (*Callicebus hoffmannsi*; ref. 41) to $2n = 16$ (*Callicebus lugens*; ref. 42). However, in this case, detailed cytogenetic analysis by

using FISH shows that despite the diversity and the rearranged karyotype, the synteny of 11 human chromosomes is maintained intact in *Callicebus callicebus* (43). At this point, it is therefore hard to conclude on the potential generality of chromosome-specific regulatory functions.

Our hypothesis is that POF is involved in a chromosome 4-specific gene regulation mechanism. This control may include the stimulation of gene expression in a highly heterochromatinized environment. Such a mechanism may explain, to some extent, the haplosufficiency of chromosome 4. Recently, a systematic analysis of the *Caenorhabditis elegans* genome, by using the RNAi technique, has shown that genes of similar function cluster in distinct regions of individual chromosomes, suggesting that different chromosomes and regions of the genome may be specialized for particular functions (44). In this context, it should be noted that similar observations were made also in *Drosophila* by Hochman (18), who concludes that both the X and the fourth chromosome are highly enriched in mutations, resulting in pupal lethality. Further work on POF has the potential to contribute to our understanding of chromosome regulatory complexes, their composition, function, and evolution.

We thank K. Yoshida for providing the *D. ananassae* cDNA library; M. Kuroda for anti-MSL antibodies; S. Åström for the *mus309* mutant stocks; I. Dacklin for injecting the *P[w+ DaPof.EYFP]* construct; and V. Pirrotta, D. Roberts, A. Saura, and S. Tuck for comments on the manuscript. This work was supported by grants from the Swedish Research Council and the Åke Wiberg, Carl Tryggers, and Philip Sörensen Foundations (to J.L.).

- Akhtar, A. (2003) *Curr. Opin. Genet. Dev.* **13**, 161–169.
- Park, Y. & Kuroda, M. I. (2001) *Science* **293**, 1083–1085.
- Pannuti, A. & Lucchesi, J. C. (2000) *Curr. Opin. Genet. Dev.* **10**, 644–650.
- Franke, A. & Baker, B. S. (1999) *Mol. Cell* **4**, 117–122.
- Stuckenholz, C., Kageyama, Y. & Kuroda, M. I. (1999) *Trends Genet.* **15**, 454–458.
- Smith, E. R., Pannuti, A., Gu, W., Sturnagel, A., Cook, R. G., Allis, C. D. & Lucchesi, J. C. (2000) *Mol. Cell. Biol.* **20**, 312–318.
- Plath, K., Mlynarczyk-Evans, S., Nusinow, D. A. & Panning, B. (2002) *Annu. Rev. Genet.* **36**, 233–278.
- Ganesan, S., Silver, D. P., Greenberg, R. A., Avni, D., Drapkin, R., Miron, A., Mok, S. C., Randrianarison, V., Brodie, S., Salstrom, J., et al. (2002) *Cell* **111**, 393–405.
- Wang, J., Mager, J., Chen, Y., Schneider, E., Cross, J. C., Nagy, A. & Magnuson, T. (2001) *Nat. Genet.* **28**, 371–375.
- Mak, W., Baxter, J., Silva, J., Newall, A. E., Otte, A. P. & Brockdorff, N. (2002) *Curr. Biol.* **12**, 1016–1020.
- Larsson, J., Chen, J. D., Rasheva, V., Rasmuson Lestander, A. & Pirrotta, V. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 6273–6278.
- Locke, J. & McDermid, H. (1993) *Chromosoma* **102**, 718–723.
- Eissenberg, J. C., Morris, G. D., Reuter, G. & Hartnett, T. (1992) *Genetics* **131**, 345–352.
- Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A. & Pirrotta, V. (2002) *Cell* **111**, 185–196.
- Miklos, G. L. G., Yamamoto, M. T., Davies, J. & Pirrotta, V. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2051–2055.
- Wallrath, L., Guntur, V., Rosman, L. & Elgin, S. (1996) *Chromosoma* **104**, 519–527.
- Wallrath, L. & Elgin, S. (1995) *Genes Dev.* **9**, 1263–1277.
- Hochman, B. (1976) in *The Genetics and Biology of Drosophila*, eds. Ashburner, M. & Novitski, E. (Academic, San Diego), pp. 903–928.
- Ashburner, M. (1989) *Drosophila: A Laboratory Handbook* (Cold Spring Harbor Lab. Press, Plainview, NY).
- Muller, H. J. (1940) in *The New Systematics*, ed. Huxley, J. (Clarendon Press, Oxford), pp. 185–268.
- Krivshenko, J. D. (1955) *Proc. Natl. Acad. Sci. USA* **41**, 1071–1079.
- Lindsley, D. L. & Zimm, G. G. (1992) *The Genome of Drosophila melanogaster* (Academic, New York).
- Gloor, G. B., Preston, C. R., Johnson-Schlitz, D. M., Nassif, N. A., Phillis, R. W., Benz, W. K., Robertson, H. M. & Engels, W. R. (1993) *Genetics* **135**, 81–95.
- Beall, E. L. & Rio, D. C. (1996) *Genes Dev.* **10**, 921–933.
- Pirrotta, V. (1988) *Bio/Technology* **10**, 437–456.
- Brand, A. H., Manoukian, A. S. & Perrimon, N. (1994) in *Methods in Cell Biology*, eds. Goldstein, L. S. B. & Fyrberg, E. A. (Academic, San Diego), pp. 635–654.
- Spradling, A. C. (1986) in *Drosophila, a Practical Approach*, ed. Roberts, D. B. (IRL, Oxford), pp. 175–197.
- Svensson, M. J., Chen, J. D., Pirrotta, V. & Larsson, J. (2003) *Chromosoma* **112**, 133–143.
- Hall, T. A. (1999) *Nucleic Acids Symp. Ser.* **41**, 95–98.
- Hazelrigg, T. (2000) in *Drosophila Protocols*, eds. Sullivan, W., Ashburner, M. & Hawley, R. S. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 313–344.
- Marin, I., Franke, A., Bashaw, G. J. & Baker, B. S. (1996) *Nature* **383**, 160–163.
- Papacit, M. & Juan, E. (1998) *Chromosome Res.* **6**, 49–54.
- Russo, C. A., Takezaki, N. & Nei, M. (1995) *Mol. Biol. Evol.* **12**, 391–404.
- Podemski, L., Ferrer, C. & Locke, J. (2001) *Chromosoma* **110**, 305–312.
- James, T. C., Eissenberg, J. C., Craig, C., Dietrich, V., Hobson, A. & Elgin, S. C. (1989) *Eur. J. Cell Biol.* **50**, 170–180.
- Barigozzi, C., Dolfini, S., Fraccaro, M., Raimondi, G. R. & Tiepolo, L. (1966) *Exp. Cell Res.* **43**, 231–234.
- Sun, F. L., Cuaycong, M. H., Craig, C. A., Wallrath, L. L., Locke, J. & Elgin, S. C. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 5340–5345.
- Lindsley, D. L., Sandler, L., Baker, B. S., Carpenter, A. T., Denell, R. E., Hall, J. C., Jacobs, P. A., Miklos, G. L., Davis, B. K., Gethmann, R. C., et al. (1972) *Genetics* **71**, 157–184.
- Sorsa, V. (1988) *Chromosome Maps of Drosophila* (CRC, Boca Raton).
- John, B. & Miklos, G. B. G. (1988) *The Eukaryote Genome in Development and Evolution* (Allen & Unwin, London).
- Rodrigues, L. R., Barros, R. M., Pissinatti, A., Pieczarka, J. C. & Nagamachi, C. Y. (2001) *Cytobios* **105**, 137–145.
- Bonvicino, C. R., Penna-Firme, V., do Nascimento, F. F., Lemos, B., Stanyon, R. & Seuánez, H. N. (2003) *Folia Primatol. (Basel)* **74**, 141–149.
- Stanyon, R., Bonvicino, C. R., Svartman, M. & Seuánez, H. N. (2003) *Chromosoma* **112**, 201–206.
- Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., et al. (2003) *Nature* **421**, 231–237.
- Bone, J. R. & Kuroda, M. I. (1996) *Genetics* **144**, 705–713.