Nucleic Acids Research, 1996, Vol. 24, No. 12 **2387–2394**

The *Drosophila* Fork head factor directly controls larval salivary gland-specific expression of the glue protein gene *Sgs3*

Václav Mach[1,2,*], Kaoru Ohno[1], Hiroki Kokubo[1] and Yoshiaki Suzuki[1]

[1]National Institute for Basic Biology, 38 Nishigonaka, Myodaiji-cho, Okazaki 444, Japan and [2]Institute of Entomology, Czech Academy of Sciences, Branisovská 31, 370 05 Ceské Budejovice, Czech Republic

Received February 2, 1996; Revised and Accepted May 2, 1996

ABSTRACT

The *Drosophila* Fork head protein participates in salivary gland formation, since salivary glands are missing in *fork head* embryos. Here we show that the *fork head* encoded protein binds to an upstream regulatory region of the larval salivary gland glue protein gene *Sgs3*. Mobility shift assay in the presence of an anti-Fork head antibody demonstrated that the Fork head factor interacts with the TGTTTGC box shown to be involved in tissue-specific Sgs3 expression. Experiments employing a set of oligonucleotide competitors revealed that Fork head binding was prevented by the same single base substitutions that were previously shown to interfere with the TGTTTGC element function *in vivo*. Furthermore, the anti-Fork head antibody bound to >60 sites of polytene chromosomes, including the puffs of all *Sgs* genes and Fork head protein was detected in the nuclei of salivary glands of larvae of all examined stages. These data provide experimental evidence for the hypothesis that the protein encoded by the *fork head* gene is required initially for salivary gland formation and is utilized subsequently in the control of larval genes specifically expressed in this organ.

INTRODUCTION

The insect labial glands, namely the salivary glands of *Drosophila* and the silk glands of the silkworm *Bombyx mori*, provide a suitable model for the study of tissue-specific gene expression, since they specifically and abundantly express several genes coding for secreted proteins (see 1,2 for reviews). We have noticed that the factor SGF-1 controlling specific transcription of the sericin-1 gene in the middle silk glands of *Bombyx* larvae (3–5) is also expressed in the developing silk glands (6). Therefore, the SGF-1 protein may be required initially for the development of silk glands and subsequently utilized in the control of genes coding for silk proteins (5). If confirmed, this hypothesis may provide a useful starting point in our attempts to understand the role of SGF-1 encoded protein in mechanisms leading to tissue-specific differentiation. There are, however, no data directly confirming SGF-1 role in silk gland formation.

The *Bombyx* SGF-1 factor (5) is a homologue of the protein encoded by the *Drosophila* region-specific homeotic gene *fork head* (7). The *Drosophila* Fork head protein must have a function in salivary gland formation, since salivary glands are missing in *fork head* embryos, whereas the labial segment is not otherwise affected (7,8). The salivary glands of Diptera and the silk glands of Lepidoptera are likely to be homologous organs (9,10). One can reason that it should be more feasible to search for *fork head* downstream target(s) in the salivary glands of *Drosophila* larvae, rather than trying to establish SGF-1/Fork head function in the developing silk glands of *B.mori*. An encouraging fact is that, when introduced into *Drosophila*, the *Bombyx* silk genes are specifically expressed in the salivary glands of transformed *Drosophila* larvae (11). It was tempting to speculate that the conserved regulatory mechanisms underlying this specific expression might include the Fork head protein.

The salivary glands of *Drosophila* coordinately express a set of seven *Sgs* genes coding for salivary gland secretion (or glue) proteins. The transcription of *Sgs* genes starts in the middle of the third larval instar and ceases abruptly at the time of puparium formation, when the glue is expectorated. In the case of the *Sgs3* gene, several *cis*-acting control elements have been dissected (see 1 for review). Among them, the proximal element (spanning the position from –130 to –56 upstream of *Sgs3*) is sufficient for tissue- and stage-specific low level expression of the *Sgs3* gene, perhaps together with specific sequences around the nucleotide +1 (12a,b). Fine mutational analysis using the transient expression assay revealed two distinct sequences within the proximal element (13). Both sequences are indispensable for the proximal element activity and presumably bind two different protein factors. We noticed that the SGF-1/Fork head cognate sequence SA contains the TGTTTGC box (3–5), which is also crucial for the function of the *Sgs3* proximal element (13).

Here we provide data strongly suggesting that the *Drosophila* Fork head protein interacts with the proximal element of the salivary gland glue protein gene *Sgs3* and regulates its tissue-specific expression. We also show that the Fork head protein is expressed in the appropriate tissue and is bound to many loci including the *Sgs* genes on polytene chromosomes. Thus *Sgs3* is the first known non-embryonic downstream target of the *fork head* encoded

* To whom correspondence should be addressed at: Institute of Entomology, Czech Academy of Sciences, Branisovská 31, 370 05 Ceské Budejovice, Czech Republic

protein and Fork head is so far the only identified tissue specifier of glue protein gene expression. Our observation, together with the fact that *fork head* embryos do not develop salivary glands (8), directly supports the hypothesis that the Fork head factor is required for both salivary gland formation and for manifestation of salivary gland-specific genes later in development.

MATERIALS AND METHODS

Developmental staging

A population of Canton S wild-type flies was maintained in small cages at 22°C. Adult flies were allowed to lay eggs on periodically replaced agar plates (1.5% w/v agar, 10% w/v glucose, 10% w/v yeast extract, live bakers yeast). To obtain staged larvae, the flies were provided with a fresh plate for 1 h. The dish was removed and larvae cultivated at 25°C. The second instar larvae were collected 60 h after egg deposition. The late third instar larvae were allowed to migrate out of food, but not to expel the glue proteins, before they were collected. Animals that had everted their spiracles were avoided. Such larvae were ~110–116 h old. The white prepupae were collected shortly after glue expectoration, when the animals already displayed pupal morphology, but before significant darkening of the cuticle occurred. For western blot experiments, 8–16 h-old embryos were collected and dechorionated using established procedures (14).

Antibody preparation

The antibody was originally prepared for the study of the *Bombyx* SGF-1/Fork head factor. The sequence of the *Bombyx* SGF-1/Fork head protein (5) was searched for local hydrophilicity and flexibility extremes (15). From several candidates, the peptide CFLRRQKRFKDEKKETLR was chosen for antibody production, since it maps within a critical part of the Fork head DNA binding domain (16). A branched form of this peptide was synthesized commercially (Iwaki Glass Corp., Funabashi City, Japan) and used to immunize a young New Zealand white male rabbit by standard procedures (17; unit 11.12.3). The immune serum Ab67 obtained after the third boost contained antibodies specifically reacting with the *Bombyx* SGF-1/Fork head 40 and 41 kDa proteins, as determined by western blot analysis. The immune peptide is conserved in the *Drosophila* Fork head factor, except for three peripheral amino acids (underlined). There is little similarity between the immune peptide and the other *Drosophila* Fork head domain-containing proteins (18). The specific interaction of the Ab67 serum with the *Drosophila* Fork head protein was determined by western blot analysis (Fig. 1). If not stated otherwise, unpurified serum was used for experiments described in this study.

Western blotting

Dechorionated embryos or manually dissected organs were mixed with 9 vol 1× SDS sample buffer (17; unit 10.2.17) and heated at 100°C for 5 min. Extracts were briefly homogenized in a manual homogenizer and cleared by centrifugation at 15 000 r.p.m. for 10 min. Proteins recovered in the supernatant were quantified by the Sigma P5656 kit. Samples (20 µg/lane) were subjected to electrophoresis in an 8% SDS–PAGE gel (17; unit 10.2.). Proteins were transferred on 0.2 micron PVDF membrane (BioRad), using the tank transfer system (150 mA, 50 V, overnight; ref. 17, unit 10.8); transfer buffer included 0.1% SDS. Blots were incubated with the anti-Fork head antibody Ab67 (diluted 1:5000–1:50 000), followed by goat anti-rabbit IgG conjugated to horseradish peroxidase. The methods were as described in the protocol supplied with the ECL western blotting detection kit (Amersham Co.); the ECL system was also used for signal visualization.

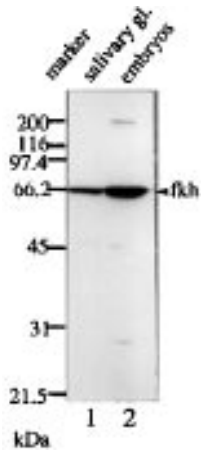

Figure 1. Western blot analysis using the Ab67 anti-Fork head serum. Proteins were extracted from salivary glands of the crawling third instar larvae (lane 1) or from 8–16 h-old embryos (lane 2). Polypeptides were separated on an 8% SDS–PAGE gel and transferred to PVDF membrane. The blot was incubated with the Ab67 serum diluted to 1:20 000. The arrow indicates the position of the presumed Fork head protein. Wide range protein markers (BioRad) were separated on the same gel, blotted and the corresponding piece of the membrane was stained with Coomassie Brilliant Blue.

Nuclear extract preparation

Nuclear extracts were prepared by a modification of the method of Georgel *et al.* (19). Salivary glands from wandering larvae were hand-dissected in Ringer's solution and stored at –70°C. All subsequent steps were performed at 4°C. 200–300 pairs of glands were added to 0.4 ml ice-cold buffer A (10 mM HEPES, pH 7.9, 1 mM $CaCl_2$, 300 mM sucrose, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMFS, 0.5 µM pepstatin A and 0.5 µM leupeptin) and homogenized by ~30 strokes of hand-held loosely fitting glass pestle. The homogenate was transferred into an Eppendorf tube together with additional 0.2 ml buffer A. The mixture was centrifuged in a Beckman JA18 rotor at 5000 r.p.m. for 5 min and the sediment was resuspended in 20 µl buffer B (10 mM HEPES, pH 7.9, 30 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% v/v glycerol, 0.5 mM DTT, 0.5 mM PMFS, 0.5 µM pepstatin A and 0.5 µM leupeptin). Nuclear proteins were extracted by slowly adding 7 µl buffer C (buffer B containing 1.6 M KCl) and gently mixing for 30 min. Afterwards, the homogenate was cleared in a Beckman JA18 rotor at 15 000 r.p.m. for 30 min, and the supernatant was recovered and dialyzed 2×90 min against 100 ml buffer D (20 mM HEPES, 100 mM KCl, 20% v/v glycerol, 1 mM EDTA, 0.5 mM DTT and 0.5 mM PMFS). The nuclear extracts

were stored at –70°C. A standard preparation yielded ~75 µg of nuclear extract at a concentration of 2.5 µg/µl.

Electrophoretic mobility shift assay and antibody reaction

The oligonucleotides used as probes and competitors in this assay are described in Figure 2 and its legend. The reactions were incubated at 4°C for 30 min and the mixture usually contained 40 µg/ml salivary gland nuclear extract, 10 mM HEPES, pH 7.9, 100 mM NaCl, 10 mM EDTA, 1 mM DTT, 2.5% Ficoll 400, 2.5 mg/ml BSA, 4 µg/ml tRNA, 4 µg/ml poly(dI–dC). Reaction mixture was aliquoted and the remaining components were added to obtain the final volume of 10 µl containing 5 fmol probe. For competition experiments, a molar excess of the competing oligonucleotide was added 15 min prior to the probe. Similarly, for the antibody reaction, the probe was added 15 min after the antibody. In some experiments, the antibody was pre-incubated with the immune or non-immune peptide for 15 min at room temperature and then added to the reaction. Following incubation, the mixture was analyzed as described (20).

Whole mount immunohistochemistry analysis

Whole mount immunohistochemistry was performed as described (21). Organs were dissected out in Ringer's solution. Fixed and blocked samples were incubated overnight at 4°C with the Ab67 anti-Fork head antibody (1:500 dilution), followed by goat anti-rabbit IgG conjugated to horseradish peroxidase. Samples were stained with the Vector substrate DAB kit and cleared in 70% glycerol in PBS.

Immunostaining of polytene chromosomes

The polytene chromosomes were prepared, stained with antibody and counterstained essentially as described (22). The squash chromosome preparations were incubated overnight at 4°C with the Ab67 anti-Fork head serum (1:400 dilution), followed by goat anti-rabbit IgG coupled to rhodamine (Cappel). Samples were inspected using the Olympus BX-50 fluorescence microscope equipped with MWIG cube, photographed, counterstained with Giemsa and mounted in Eukitt.

RESULTS

The *Drosophila* Fork head protein binds *in vitro* to the proximal element of the *Sgs3* gene

The presence of both *fork head*-encoded protein and mRNA in embryonic salivary glands and some other embryonic organs was demonstrated (7), whereas *fork head* expression in larval salivary glands was studied only at the mRNA level (23). In a series of western blot experiments, we examined the presence of the Fork head protein in salivary glands of the third instar wandering larvae (Fig. 1). The antibody reacted with extracts prepared from both the late stage embryos and larval salivary glands and a single band was detected using antibody dilutions of 1:10 000–1:50 000 (Fig. 1 and data not shown). The observed molecular mass of this protein was 65 kDa, which is 11 kDa more than calculated from sequence of *fork head* cDNA (7). Anomalous migration of some proteins in SDS–PAGE has been occasionally reported (e.g. 24) and offers an explanation for the observed difference.

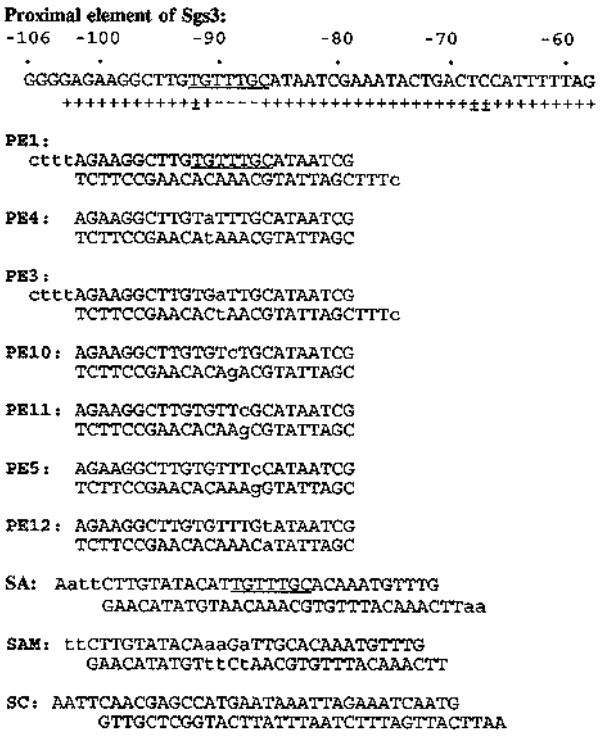

Figure 2. Sequences of the proximal element and of oligonucleotides used in this study. The conserved TGTTTGC box is underlined in the proximal element sequence and in the upper strands of PE1 and SA oligonucleotides. Proximal element of *Sgs3*: the sequence of the *Sgs3* proximal element is from Todo *et al.* (13). The authors of the above study mutated individual bases within the proximal element and tested each mutant for its ability to direct salivary glands expression of alcohol dehydrogenase reporter using the transient expression assay and histochemical staining. + Indicates that staining was seen in the salivary glands of some injected animals; – that no staining was seen; and ± that staining was seen only using a more sensitive assay. More details can be found in Todo *et al.* (13). PE1, PE4, PE3, PE10, PE11, PE5, PE12: double-stranded oligonucleotides based on the proximal element sequence. Small letters indicate nucleotides not present in the wild-type sequence. The PE1 is a wild-type oligonucleotide and was used as a probe. The substitutions in its termini were introduced to facilitate labeling by Klenow enzyme. The remaining PE oligonucleotides are similar to PE1, but contain single base substitutions within the conserved TGTTTGC box. These substitutions correspond to point mutations introduced by Todo and co-workers (13). SA, SAM: these oligonucleotides are derived from the SA site of the *Bombyx* sericin-1 gene (4). SA is a wild-type oligonucleotide efficiently interacting with the *Bombyx* SGF-1/Fork head factor. SAM is a mutated version of SA and does not efficiently interacts with the SGF-1/Fork head. Both oligonucleotides are the same as used by Mach and co-workers (5). SC: this oligonucleotide, originally used by Matsuno and co-workers (4), does not contain the TGTTTGC box and served as a heterologous competitor.

Our previous studies revealed that the cognate site SA for the *Bombyx* SGF-1/Fork head factor contains a central TGTTTGC sequence (3–5). The TGTTTGC box also occurs in several DNA elements recognized by the Hepatocyte nuclear factor 3, which is a mammalian member of the *fork head* family (25). We have noticed that the TGTTTGC box is also present in the proximal element of the *Sgs3* gene near nt –90 (Fig. 2). This particular TGTTTGC motif overlaps with the binding site for a regulatory protein, since the sequence harbors five of the seven mutations disturbing the proximal element function (13; see also Fig. 2). The remaining two mutations affect nt –67 and –68 and most likely define cognate site of a different protein factor (13). To

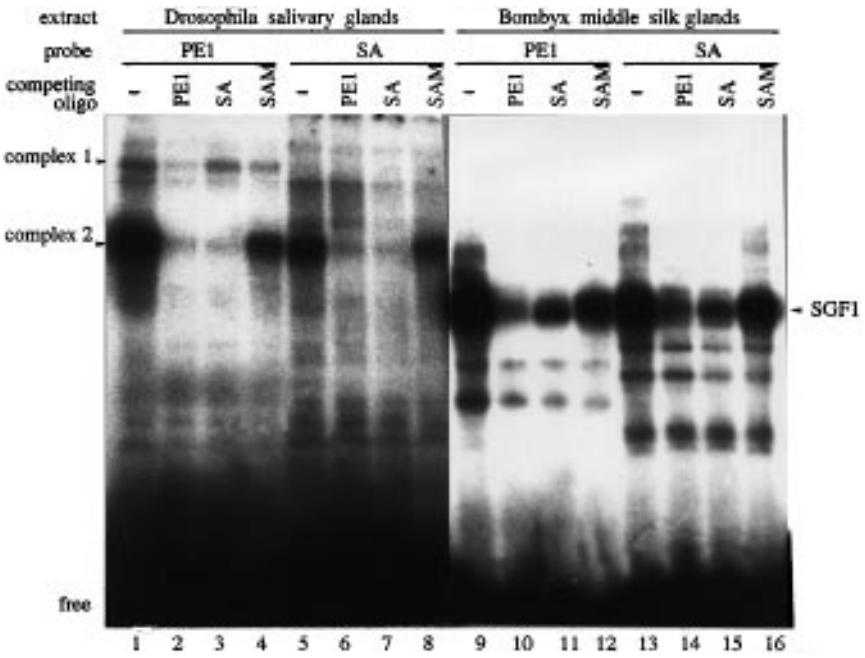

Figure 3. PE1 and SA oligonucleotides produce similar complexes in both the *Bombyx* and *Drosophila* extracts. Lanes 1–8: the electrophoretic mobility shift assay was performed with *Drosophila* salivary glands extract as described in Materials and Methods. Lanes 9–16: the assay was performed with 100µg/ml of *Bombyx* middle silk glands extract (prepared as described in 5), 10 mM HEPES, 100 mM NaCl, 10 mM EDTA, 1 mM DTT, 1 mg/ml BSA, 20µg/ml poly(dI–dC) and 20 µg/ml tRNA. The PE1 (lanes 1–4 and 9–12) and SA oligonucleotides (lanes 5–8 and 13–16) were used as probes. Indicated reactions contained 50 times molar excess of PE1, SA or SAM oligonucleotides. The arrows show the positions of complex-1 and complex-2 in the salivary gland extract and the position of the SGF-1/Fork head complex in the silk glands extract. Oligonucleotides used in this experiment are described in Figure 2 and its legend. Oligos: PE1, proximal element (SGS3); SA, SA site (sericin-1); SAM, mutated SA site.

distinguish these two functional domains within the proximal element, the sequence from –76 to –102, harboring the TGTTTGC box, will be further called the proximal element (–76,–102).

The presence of the functionally important TGTTTGC box within the proximal element (–76,–102) led us to speculate that the unknown factor interacting with this sequence (13) must be *Drosophila* Fork head. This assumption was verified using the electrophoretic mobility shift assay. Nuclear extracts were prepared from salivary glands of the third instar crawling larvae, since the *Sgs3* gene is intensively transcribed at this stage of development (26). The PE1 (PE stands for proximal element) double-stranded oligonucleotide, containing bases –76 to –102 upstream of the *Sgs3* gene (Fig. 2), was used as a probe. An initial electrophoretic mobility shift assay experiment revealed two specific bands, which we named complex-1 and complex-2 (data not shown, but see results in Fig. 3).

The DNA binding domains of the *Drosophila* Fork head and *Bombyx* SGF-1/Fork head factors are highly conserved (5) and should recognize similar DNA sequences. Therefore, if the proximal element (–76,–102) contains a binding site for the *Drosophila* Fork head, this sequence should also react with the *Bombyx* Fork head homologue SGF-1. Similarly, the *Bombyx* SGF-1/Fork head cognate site SA should be bound by the Fork head protein present in the *Drosophila* salivary glands nuclear extract. In other words, oligonucleotides based respectively on the proximal element (–76,–102) and SA site sequences (Fig. 2) should reveal similar complexes in the electrophoretic mobility shift assay performed with the same extract. This assumption was fully confirmed (Fig. 3). The SA oligonucleotide produced a major complex co-migrating with the complex-2 of PE1 oligonucleotide in the *Drosophila* extract (Fig. 3, compare lane 5 with 4). The effects of oligonucleotide competitors strongly suggest that the major complexes formed by the PE1 and SA probes include the same protein (Fig. 3, lanes 1–8; refer to Fig. 2 and its legend for details concerning competing oligonucleotides). Similarly, both DNAs produced SGF-1 complex in the silk gland extract (Fig. 3, lanes 9–16). The identity of the SGF-1 complex was confirmed by the electrophoretic mobility shift assay using the pure renatured SGF-1 40 kDa protein (see ref. 5 for SGF-1 purification and the SA oligonucleotide data; PE1 data are not shown). Taken together these data indicate that complex-2 is formed by the *Drosophila* Fork head protein.

To confirm the identity of complex-2 we have tried an independent and more direct experiment. Pre-incubation of nuclear extract with increasing amounts of anti-Fork head Ab67 antibody resulted in the gradual disappearance of complex-2, without it being replaced by a significant ‘hypershifted’ band (Fig. 4A), whereas addition of an unrelated rabbit serum had no effect (data not shown). The Ab67 polyclonal antibody was directed against a synthetic peptide derived from a region which is critical for function of the conserved Fork head/Hepatocyte nuclear factor 3 DNA-binding domain (16). Therefore, addition of the antibody prevented Fork head-DNA binding instead of resulting in formation of a ‘hypershifted’ complex. The antibody effect was competed out by the presence of the immune peptide (Fig. 4B) but not by similar or larger amounts of an unrelated peptide (data not shown). We have concluded that the complex-2 protein is the *Drosophila* Fork head factor.

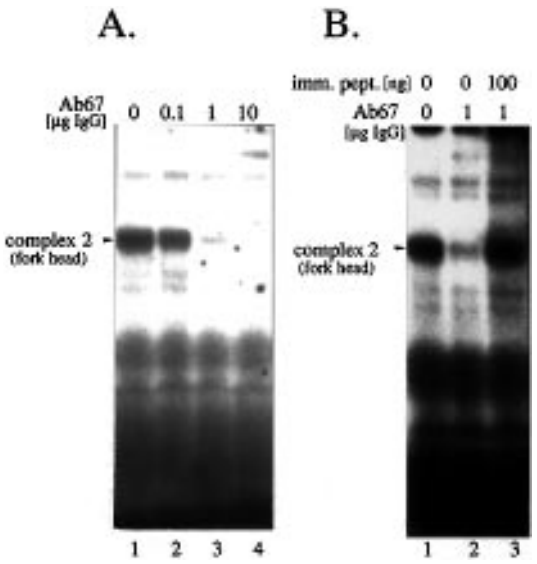

Figure 4. The anti-Fork head antibody Ab67 specifically interacts with the complex-2 protein. Purified IgG used for this experiment was obtained from the Ab67 serum by chromatography on an Affi Gel Blue CM column (BioRad) followed by ammonium sulphate precipitation. (**A**) Electrophoretic mobility shift assay reactions contained 400 ng of *Drosophila* salivary glands nuclear extract in the volume of 10 µl. Reactions were pre-incubated with increasing amounts of Ab67 antibody. Lanes 1–4 contained 0, 0.1, 1 and 10 µg Ab67 IgG respectively. (**B**) Competition of the antibody effect by the immune peptide. Reactions contained no Ab67 IgG and no immune peptide (lane 1), 1 µg IgG and no immune peptide (lane 2) and 1 µg IgG and 100 ng immune peptide (lane 3). Complex-2, formed by the Fork head protein, is indicated by an arrowhead.

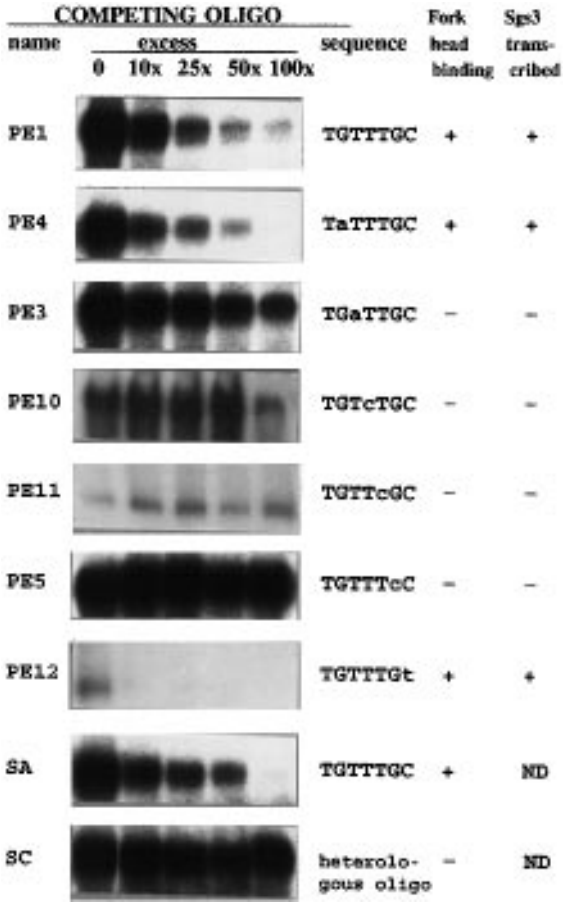

Figure 5. Correlation between Fork head binding and the proximal element activity. Fork head factor binding to various oligonucleotides was assessed by competitive electrophoretic mobility shift assay performed in salivary glands extract. Only complex-2 (Fork head) is shown. Reactions contained the wild-type PE1 probe and indicated molar excess of competing oligonucleotides. The column 'sequence' shows important features of the oligonucleotide used as competitor: the PE4, PE3, PE10, PE11, PE5 and PE12 oligonucleotides differ from PE1 by indicated single base substitutions within the TGTTTGC box. The SA oligonucleotide also contains the TGTTTGC box, while the SC oligonucleotide served as a heterologous control competitor. A full description of competing oligonucleotides is given in Figure 2 and its legend. The column '*fork head* binding' describe the effects of competing oligonucleotide; + means that the oligonucleotide efficiently competed with the PE1 probe for the Fork head factor and therefore the introduced substitution did not affect Fork head binding; – that the oligonucleotide did not efficiently compete for the Fork head factor and therefore the respective mutation prevented Fork head binding. The column '*Sgs3* transcribed' describes the effects of the corresponding single base substitutions within the TGTTTGC box on the proximal element activity, as assessed by the transient expression assay (ref. 13; see also Fig. 2); + means that the mutation does not affect the proximal element activity; – that the authors of the above paper evaluate this mutation as fully effective; ND, not determined.

Nucleotides critical for the proximal element function are also required for Fork head binding

We have analyzed the relationship of the Fork head protein with the unknown factor interacting with the TGTTTGC box *in vivo* (13). Provided that this factor is Fork head, the nucleotides critical for the proximal element (–76,–102) function in the transient expression assay (13) must also be indispensable for the *in vitro* Fork head binding to this element. We constructed a set of oligonucleotides based on the proximal element sequence, each containing a single base substitution within the TGTTTGC box (PE4, PE3, PE10, PE11, PE5, PE12; Fig. 2). The substitutions matched the point mutations introduced by Todo and co-workers (13). We tested the ability of these modified oligonucleotides to compete with the wild-type PE1 oligonucleotide for the Fork head protein (Fig. 5). As expected, the residues affecting Fork head–DNA interaction (Fig. 5) are the same which were shown to be critical for the proximal element function in the transient expression assay (13; see also Fig. 2). On the other hand, if a mutation within the TGTTTGC box does not have an effect on reporter construct transcription (13) it does not influence the Fork head binding (Fig. 5). These data (Fig. 5), combined with the observation of Todo and co-workers (13), strongly suggest that Fork head protein both recognizes TGTTTGC box *in vitro* and stimulates Sgs3 transcription *in vivo*.

Fork head protein is present in salivary glands of several stages and in a limited number of other tissues

The *Sgs3* gene is specifically expressed in the salivary glands of middle to late third instar larvae (26), whereas we found the Fork head protein in the salivary glands of not only the third instar larvae (Fig. 6B) but also of the second instar larvae as well as of the white prepupae (Fig. 6A and C respectively). Therefore it is unlikely that the Fork head factor itself determines the time course of *Sgs3* expression. On the other hand, *fork head* encoded protein is a good candidate for tissue specifier of glue gene expression, since Kuzin *et al.* (23) detected the presence of *fork head* mRNA only in some tissues of third instar larvae. The protein distribution shown in Figure 6 closely mirrors the published mRNA data (23). Besides the salivary glands, we noticed strong Ab67 staining of nuclei in the midgut caeca, hindgut–midgut boundary and

Figure 6. Developmental expression (A–C) and tissue distribution (D–F) of the Fork head protein. Organs of *Drosophila* larvae were immunostained with the Ab67 anti-Fork head serum. Nuclear localized Fork head protein is evenly distributed in the salivary glands of the second (**A**) and late third instar larvae (**B**), whereas the signal already starts to fade away from the posterior part of the salivary glands in white prepupa (**C**). Scale bars indicate 0.1 mm. Besides salivary glands, the Fork head protein is strongly expressed in midgut caeca (**D**), at the midgut-hindgut boundary (**E**) and in Malpighian tubules (**F**) of the wandering third instar larvae. There was no discernible staining of other parts of the gut, with a possible exception of a weak signal in the anterior midgut. We have also occasionally observed a faint staining of fat body contaminating our preparations (not shown). (*b.*) border between midgut and hindgut; (*h.*) hindgut; (*m.*) midgut; (*m.c.*) midgut caeca; (*m.t.*) Malpighian tubules; (*s.g.*) salivary glands.

Malpighian tubules (Fig. 6D, E and F respectively). We restricted our study to the gut and adjacent organs and could not, therefore, confirm Fork head presence in the lymphoid glands.

Fork head protein associates with the loci of *Sgs* genes on polytene chromosomes

Immunostaining of polytene chromosomes is used to detect transcription factors associated *in vivo* with specific chromosomal domains (22). We found that the Ab67 anti-Fork head antibody bound to >60 sites of polytene chromosomes prepared from crawling third instar larvae (Fig. 7). The high number of observed signals (but not their distribution) resembled the situation found with the Polycomb and serendipity proteins (27,28). There are at least two reasons to believe that the observed signals are specific, except perhaps for the very faint ones. First, most of the immunostained bands were reproducibly observed in a large number of independent samples, occasional differences were probably caused by chromosome preparation. Secondly, the distribution of the antibody binding sites was not random, since large portion of the well-defined signals mapped within the intermolt puffs (Fig. 7, compare B and E with C and F). The immunostained loci also included the five puffs associated with *Sgs* genes. In fact, the *Sgs* loci repeatedly revealed noticeably strong signals, with a possible exception of 90C (Fig. 7 and data not shown).

In conclusion, the Fork head factor co-localizes *in vivo* with all known loci of glue protein genes, including *Sgs3*.

DISCUSSION

Direct regulation of glue protein genes by the Fork head factor

We have demonstrated *in vitro* that the *Drosophila* Fork head factor directly interacts with the proximal element (–76,–102) of the *Sgs3* gene (Figs 3 and 4). Except for the experiment shown in Figure 3, reliability of the data supporting our conclusions strictly depends on the antibody specificity. The antibody was originally prepared to study the *Bombyx* SGF-1/Fork head factor, but the high conservation of the immune peptide (see Materials and Methods) allowed us to use the antibody for the study of the *Drosophila* Fork head factor as well. The specificity of the antibody reaction was clearly demonstrated—we observed a single band on western blot using a wide range of antibody dilutions (Fig. 1 and data not shown), the immune serum reacted with a nuclear protein as expected (Fig. 6 and ref. 7), and the tissue distribution of this protein agreed exactly with the published

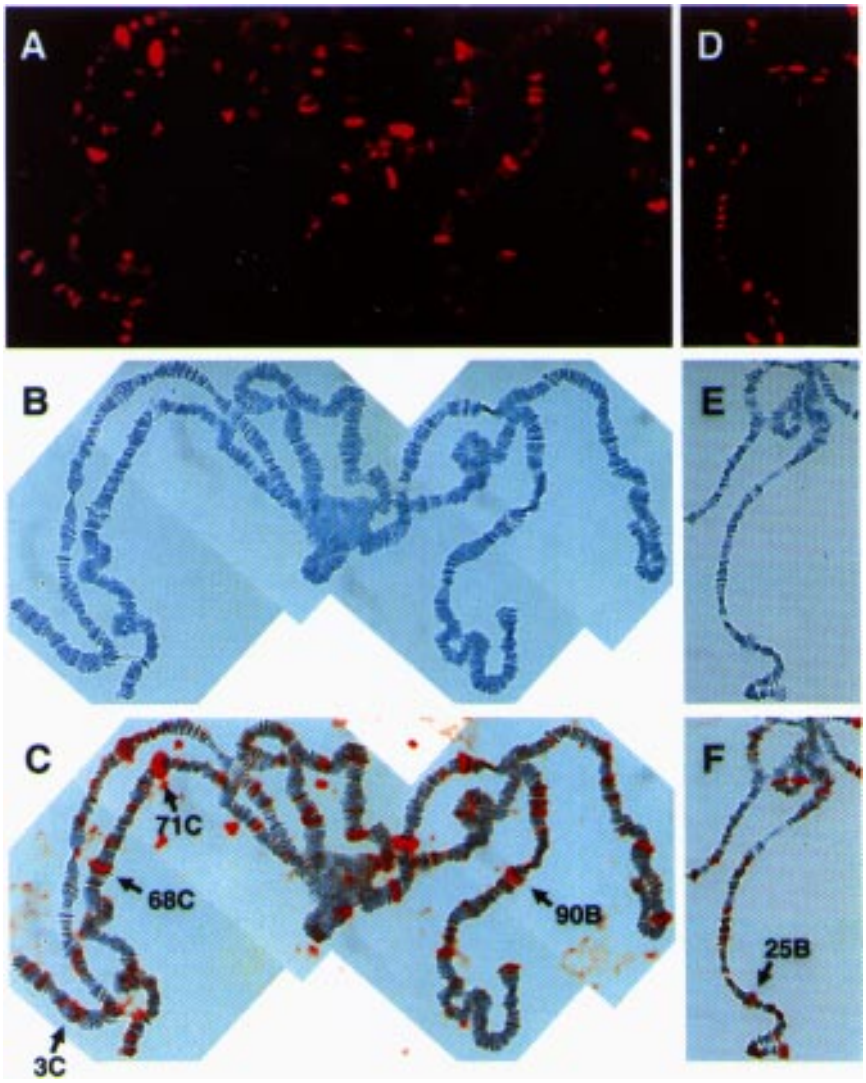

Figure 7. The *Sgs* loci are immunostained with the anti-Fork head antibody. (**A**) A representative sample of immunostained polytene chromosomes prepared from the salivary glands of wandering third instar larvae. The bands of red fluorescence correspond to the binding sites of Ab67 anti-Fork head antibody. The photograph was obtained using an Olympus BX-50 fluorescence microscope equipped with MWIG cube and was taken prior to the Giemsa staining. (**B**) The same nucleus counterstained with Giemsa. This photograph was compared with published polytene chromosome maps (35,36) and used to determine the positions of observed signal. (**C**) A composite picture prepared with the Fuji HC-1000 microscope-CCD camera-computer system and the Adobe Photoshop software. The cytological localization of individual *Sgs* genes is from Lindsley and Zimm (1992) and from the FlyBase updates (gopher://ftp.bio.indiana.edu/Flybase). *Sgs* genes are associated with the following intermolt puffs: 3C (*Sgs4*), 25B (*Sgs1*), 68C (*Sgs3*, *Sgs7* and *Sgs8*), 71C (*Sgs6*) and 90B (*Sgs5*).

mRNA data (Fig. 6 and ref. 23). In addition, the antibody interfered with the function of the DNA binding domain as anticipated (Fig. 4A and ref. 16) and this effect was specifically competed out by the immune peptide (Fig. 4B and data not shown).

Another question is whether the *fork head* encoded protein functions as an *in vivo* activator of the *Sgs3* gene. Animals homozygous for all known *fork head* alleles die during embryonic development (8) and transformation with a 15.3 kb fragment containing the cloned *fork head* gene is not sufficient to rescue this effect (7). Therefore P-element rescue experiments using homozygous *fork head* larvae must be preceded by more detailed characterization of the *fork head* gene itself. For this reason we have chosen a less direct, but more feasible, approach by showing that the *in vitro* Fork head binding to the proximal element strictly correlates with the element activity *in vivo* (Fig. 5) and that the Fork head protein co-localize with the *Sgs* loci on polytene chromosomes (Fig. 7). These data strongly suggest that Fork head protein controls the expression of the *Sgs3* gene.

Does the Fork head protein also control other glue genes? The answer is probably affirmative, since the anti-Fork head antibody clearly and consistently bound at the polytene chromosomes loci of all *Sgs* genes (Fig. 7 and data not shown) and elements similar to the conserved TGTTTGC box occur at the upstream regions of several glue protein genes (13). Finally, in the period between submission of the initial and revised versions of the manuscript, it came to our attention that another group obtained data implicating Fork-head role in the regulation of salivary glands glue gene *Sgs-4* (G. Korge, personal communication). The analogy with the *Bombyx* silk glands is appealing. Although the Bombyx SGF-1/Fork head protein was purified as a factor interacting with the SA site of the sericin-1 gene (5), the same protein interacts with sites located both upstream and within the

first intron of the H-chain fibroin gene (20 and S. Takiya, unpublished data) and putative SGF-1 binding sites are present in regulatory regions of other silk genes.

The Fork head protein is present in a limited number of tissues and in all examined developmental stages (Fig. 6). Therefore it may be a tissue specifier of *Sgs3* gene expression, whereas another group of factors may confer the stage specificity. Several polypeptides coded by the *Broad-Complex* were shown to initiate *Sgs4* gene expression in response to increasing titre of the insect molting hormone, ecdysone (29). Although the direct control of the *Sgs3* gene by the *Broad-Complex* proteins remains to be demonstrated, it is indicated by all available data (19,29–31).

The role of Fork head protein in salivary glands

Fork head is a homeotic gene promoting region-specific development in both the posterior and anterior terminal domains of *Drosophila* embryo. The salivary glands are missing in *fork head* embryos and the Fork head protein is present in salivary glands since the formation of their placodes at the stage 11 of embryonic development (7,8). An initial impetus for this study was the need to provide experimental evidence supporting the hypothesis that the Fork head protein is initially required for insect labial gland formation and subsequently utilized in the control of larval genes specifically expressed in the same organ. Indeed, we have demonstrated that the *fork head* encoded protein is required for the expression of the *Drosophila* salivary gland-specific gene *Sgs3* and perhaps also other glue protein genes (see above). What remains unclear is the identity of the other (so far unknown) genes probably controlled by the *fork head* encoded protein in salivary gland. We speculate that these genes are salivary gland-specific or participate on gland formation. According to this model, the *fork head* encoded protein in insect labial glands recognizes the TGTTTGC box (or similar sequences) localized in regulatory regions of such genes. The salivary gland-specific restriction of the Fork head *in vivo* binding to a subset of its cognate sites may be achieved by presence of a co-factor, e.g. the protein interacting next to TGTTTGC box within the proximal element (13). One possibility is that Fork head binding would possibly turn target genes to a 'transcription allowed' state but the actual expression would be delayed until appearance of additional regulatory factors. Such a 'transcription allowed' state may be acquired by changes in chromatin organization, since the three-dimensional structure of the conserved Fork head/Hepatocyte nuclear factor 3 DNA-binding domain resembles histone H5 (16) and the Hepatocyte nuclear factor 3 is implicated in the formation of precisely positioned nucleosomes (33). Although speculative, this model may conveniently explain some observations, including the presence of Fork head protein in Sgs3 non-expressing stages (Fig. 6A and ref. 7). Another example are the chromosomes from salivary glands of ecdysone deficient larvae, which forms the puffs of *Sgs3* and *Sgs4* genes, but the genes themselves remain silent (cited from 34). This would be expected if the Fork head protein is needed for the puff formation, whereas simultaneous presence of ecdysone-inducible proteins (see Discussion above) is required for actual transcription.

ACKNOWLEDGEMENTS

We are grateful to Drs A. Nose and T. Umemiya for their kind help with the *Drosophila* culture and embryos collection, Dr Günter Korge for communicating results prior to publication and Dr P. Svácha for useful discussion. This research was supported in part by grants-in-aid for Research of Priority Areas and for Japanese Society for the Promotion of Science Fellows from the Ministry of Education, Science, and Culture of Japan and by the grant 301/96/0153 of the Grant Agency of Czech Republic.

REFERENCES

1 Meyerowitz, E.M., Raghavan, K.V., Mathers, P.H. and Roark, M. (1987) *Trends Genet.*, **3**, 288–293.
2 Suzuki, Y. (1994) *Int. J. Dev. Biol.*, **38**, 231–235.
3 Matsuno, K., Hui, C., Takiya, S., Suzuki, T., Ueno, K. and Suzuki, Y. (1989) *J. Biol. Chem.*, **264**, 18707–18713.
4 Matsuno, K., Takiya, S., Hui C., Suzuki, T., Fukuta, M., Ueno, K. and Suzuki, Y. (1990) *Nucleic Acids Res.*, **18**, 1853–1858.
5 Mach, V., Takiya, S., Ohno, K., Handa, H., Imai, T. and Suzuki, Y. (1995) *J. Biol. Chem.*, **270**, 9340–9346.
6 Kokubo, H., Takiya, S., Mach, V. and Suzuki, Y. (1996) *Roux's Arch. Dev. Biol.*, in press.
7 Weigel, D., Jürgens, G., Kuttner, F., Seifert, E. and Jäckle, H. (1989) *Cell*, **57**, 645–657.
8 Jürgens, G. and Weigel, D. (1988) *Roux's Arch. Dev. Biol.*, **197**, 345–354.
9 Goldsmith, M.R. and Kafatos, F.C. (1984) *Annu. Rev. Genet.*, **18**, 443–487.
10 Turner, F.R. and Mahowald, A.P. (1977) *Dev. Biol.*, **57**, 403–416.
11 Bello, B. and Couble, P. (1990) *Nature*, **346**, 480–482.
12 a. Roark, M., Raghavan, K.V., Todo, T., Mayeda, C.A. and Meyerowitz, E.M. (1990) *Dev. Biol.*, **139**, 121–133. b. Martin, M., Giangrande, A., Ruiz, C. and Richards, G. (1989) *EMBO J.* **8**, 561–568.
13 Todo, T., Roark, M., Raghavan, K.V., Mayeda, C. and Meyerowitz, E. (1990) *Mol. Cell. Biol.*, **10**, 5991–6002.
14 Roberts, D.B. (1986) In Roberts, D.B. (ed.), *Drosophila: A Practical Approach.* IRL Press, Oxford, pp. 1–38.
15 Carmenes, R.S., Freije, J.P., Molina, M.M. and Martin, J.M. (1988) *Biochem. Biophys. Res. Comm.*, **159**, 687–693.
16 Clark, K.L., Halay, E.D., Lai, E. and Burley, S.K. (1993) *Nature*, **364**, 412–420.
17 Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) *Current Protocols in Molecular Biology.* John Wiley & Sons, Inc., New York.
18 Häcker, U., Grossniklaus, U., Gehring, W.J. and Jäckle, H. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8754–8758.
19 Georgel, P., Ramain, P., Giangrande, A., Dretzen, G., Richards, G. and Bellard, M. (1991) *Mol. Cell. Biol.*, **11**, 523–532.
20 Hui, C., Matsuno, K. and Suzuki, Y. (1990) *J. Mol. Biol.*, **213**, 651–670.
21 Patel, N.H., Condron, B.G. and Zinn, K. (1989) *Cell*, **58**, 955–968.
22 Andrew, D.J. and Scott, M.P. (1994) In Goldstein, L.S.B. and Fyrberg, E.A. (eds), *Drosophila melanogaster: Practical Uses in Cell and Molecular Biology.* Academic Press, Inc., Methods in Cell Biology Vol. 44., pp.354–371.
23 Kuzin, B., Tillib, S., Sedkov, Y., Mizrokhi, L. and Mazo, A. (1994) *Genes Dev.*, **8**, 2478–2490.
24 Takano, E., Maki, M., Mori, H., Hatanaka, N., Marti, T., Titani, K., Kannagi, R., Ooi, T. and Murachi, T. (1988) *Biochemistry*, **27**, 1964–1972.
25 Lemaigre, F.P., Durviaux, S.M. and Rousseau, G.G. (1993) *J. Biol. Chem.*, **268**, 19896–19905.
26 Andres, A.J., Fletcher, J.C., Karim, F.D. and Thummel, C.S. (1993) *Dev. Biol.*, **160**, 388–404.
27 Zink, B. and Paro, R. (1989) *Nature*, **337**, 468–469.
28 Payre, F., Noselli, S., Lefrere, V. and Vincent, A. (1990) *Development*, **110**, 141–149.
29 Kalm, von L., Crossgrove, K., Seggern, U.D., Guild, G.M. and Beckendorf, S.K. (1994) *EMBO J.*, **13**, 3505–3516.
30 Karim, F.D., Guild, G.M. and Thummel, C.S. (1993) *Development*, **118**, 977–988.
31 Crowley, T.E., Mathers, P.H. and Meyerowitz, E.M. (1984) *Cell*, **39**, 149–156.
32 Kaufmann, E., Hoch, M. and Jäckle, H. (1994) *Eur. J. Biochem.*, **223**, 329–337.
33 McPherson, C.E., Shim, E.-Y., Friedman, D.S. and Zaret, K.S. (1993) *Cell*, **75**, 387–398.
34 Lehmann, M. and Korge, G. (1995) *EMBO J.*, **14**, 716–726.
35 Lefevre, G., Jr (1976) In Ashburner, M. and Novitski, E. (eds), *The Genetics and Biology of Drosophila.* Academic Press, Inc., Vol. 1a, pp. 31–66.
36 Lindsley, D.L. and Zimm, G.G. (1992) *The Genome of Drosophila melanogaster.* Academic Press, Inc.