Function and specificity of synthetic Hox transcription factors in vivo

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Homeotic (*Hox*) genes encode transcription factors that confer segmental identity along the anteroposterior axis of the embryo. However the molecular mechanisms underlying *Hox*-mediated transcription and the differential requirements for specificity in the regulation of the vast number of *Hox*-target genes remain ill-defined. Here we show that synthetic *Sex combs reduced* (*Scr*) genes that encode the Scr C terminus containing the homedomain (HD) and YPWM motif (Scr-HD) are functional in vivo. Synthetic Scr-HD peptides can induce ectopic salivary glands in the embryo and homeotic transformations in the adult fly, act as transcriptional activators and repressors during development, and participate in protein-protein interactions. Their transformation capacity was found to be enhanced over their full-length counterpart and mutations known to transform the full-length protein into constitutively active or inactive variants behaved accordingly in the synthetic peptides. Our results show that synthetic *Scr*-HD genes are sufficient for homeotic function in *Drosophila* and suggest that the N terminus of Scr has a role in transcriptional potency, rather than specificity. We also demonstrate that synthetic peptides behave largely in a predictable way, by exhibiting *Scr*-specific phenotypes throughout development, which makes them an important tool for synthetic biology.

synthetic genes | transcriptional specificity | *Hox* genes | *Sex combs reduced* | homeotic transformations

Homeotic genes code for transcription factors that play an instrumental role in animal development by specifying the identity of body segments along the anteroposterior axis of the embryo (1–4). *Hox* genes have persisted in the animal kingdom; they are found in animals as diverse as worms and humans (5, 6) and the Homeodomain (HD), a helix-turn-helix DNA-binding domain, has been strikingly conserved in animals since before the bilaterian split (1, 7, 8). Sequence-specific binding of Hox proteins has been studied for the *Drosophila* Sex combs reduced (Scr) (9, 10), Antennapedia (Antp) (11, 12) and Ultrabithorax (Ubx) (11, 12) HDs. A consensus sequence TAATC/GC/G recognition core was identified in all of them, which alone is obviously not sufficient to confer transcriptional specificity, because it occurs statistically every kilobase in the genome. Similar sequence preferences have been identified for Deformed (Dfd) and Abdominal-B (Abd-B) (11), raising the question of how target specificity is achieved among different *Hox* paralogs.

A closer look into conserved residues outside the HD identified its amino-terminal YPWM motif that is present in almost all Hox proteins, from flies to vertebrates [with the exception of Abdominal-B (Abd-B), which has conserved only the tryptophan at position 3] (13). Extradenticle (Exd) and its mammalian homolog Pbx1 (14) were found to interact specifically with the YPWM motif of Hox proteins in vitro (15) and crystallographic analysis of a Ubx-Exd complex determined the topology of this interaction (16). Recently the link between the Antp YPWM motif and the transcriptional machinery was made through the identification of Bric-à-Brac interacting protein 2 (Bip2) (17). However, *Hox* cofactors do not suffice to entirely explain Hox specificity. The finding of cofactor independent Hox function (18) contributed to the realization that further sequences residing in the N terminus of Hox proteins might be the link for increased specificity in vivo.

In the present work we have derived synthetic *Scr* genes that encode the YPWM, HD and C terminus of the *Drosophila* Scr. The synthetic Scr-HD retained many functions of the full-length protein in vivo by participating in homeotic transformations, transcriptional regulation, and protein-protein interactions, thus reflecting to a great extent the properties of the native Scr protein. Constitutively active and inactive variants, in which threonine-6 and serine-7 of the Scr-HD have been substituted by alanines (Scr-HD_{AA}) or aspartates (Scr-HD_{DD}), respectively (19), behaved in the synthetic peptides similar as in the full-length protein, but the synthetic peptides exhibited stronger homeotic function as compared to their full-length counterpart. Moreover, we show that the synthetic peptides bind specifically to native Scr and consensus HD-binding sites in vitro and accumulate at sites of loose chromatin conformation in live salivary gland nuclei, where *Scr* is normally expressed during development (20). Taken together our results indicate that synthetic Scr peptides are functional in vivo and thus challenge the role of the N-terminal part of the endogenous fly peptide in transcriptional specificity.

BIOPHYSICS AND COMPUTATIONAL BIOLOGY

Results

Synthetic *Scr* Genes are Capable of Inducing Ectopic Salivary Glands in the Embryo. *Scr* acts as a master control regulator of salivary gland morphogenesis during embryonic development (20–25). When expressed throughout the embryo, Scr is able to induce an additional pair of salivary glands anterior to parasegment 2 (20), where the normal salivary glands form. Posteriorly its function is restricted by *teashirt* (*tsh*) and *Abdominal-B* (*Abd-B*) (26). In accordance with these findings, the wild type (*Scr*-HD_{wt}) and the constitutively active (*Scr*-HD_{AA}) synthetic genes could also induce ectopic salivary glands in the embryonic head, while the constitutively inactive variant (*Scr*-HD_{DD}) could not (Fig. 1*A*–*C*) and displayed a normal pair of salivary glands, similar to the control embryos, also treated with heat shock (Fig. 1*D*).

Misexpression of Synthetic *Scr* Genes Causes Homeotic Transformations in the Adult Head. Both the fly *Scr* gene and its functional mouse homolog *Hox-a5* (previously described as *Hox-1.3*) have been shown to induce partial antenna-to-tarsus transformations in the fly head (27, 28). To examine the ability of Scr-HD_{wt} and Scr-

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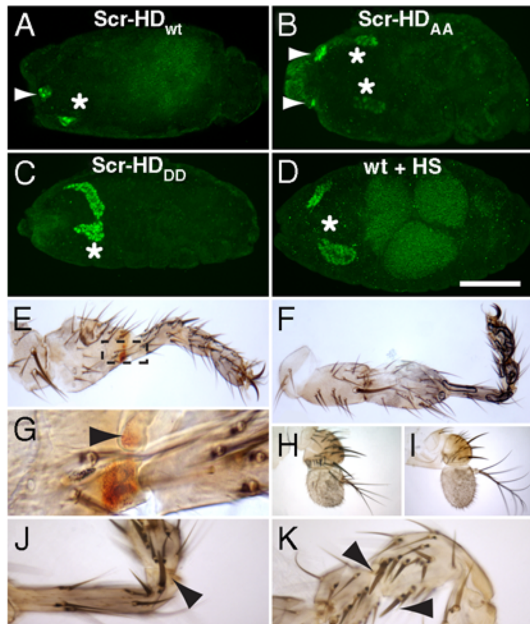

Fig. 1. Synthetic Scr peptides induce homeotic transformations in the fly (*A–C*). Expression of the synthetic genes throughout the embryo results in the formation of additional salivary glands in the cephalic region for *Scr*-HD_{wt} (*A*) and *Scr*-HD_{AA} (*B*), but not *Scr*-HD_{DD} (*C*). (*D*) Wild type embryo also treated with heat shock allows the development of one normal pair of salivary glands. Arrowheads show the ectopic—and asterisks, the normal—salivary glands. All constructs were induced using *Heat-Shock*-Gal4. Stainings are on stage 16 embryos for dCREB-A (19), a salivary gland luminal marker. Scale bar 100 μm. (*E–I*) Expression of *Scr*-HD_{wt} (*E*) and *Scr*-HD_{AA} (*F*) in the antennal disc results in complete antenna-to-tarsus transformations. (*G*) Magnification of the outlined area in (*E*) shows pigmented cells (*Arrowhead*) at the distal part of the transformed A3. (*H*) *Scr*-HD_{DD} only confers a small reduction in the size of the arista. (*I*) Wild type antenna. (*J–K*) Sex comb teeth on antennal tarsi generated by ectopic expression of *Scr*-HD_{wt} (*J*) and *Scr*-HD_{AA} (*K*) (*Arrowheads*). All transformations were generated using a *Dll*-Gal4 driver.

HD_{AA} to trigger homeotic transformations in vivo we expressed them ectopically in the antennal portion of the eye-antennal disc using the *Distalless* (*Dll*) enhancer (29). Transformation of the third antennal segment (A3) and the arista to a fully grown tarsus (Fig. 1*E* and *F*, respectively) was observed. The inactive mutant (Scr-HD_{DD}) could only induce a reduction in the size of the arista (Fig. 1*H*) as compared to the wt antenna (Fig. 1*I*). The ectopic tarsi generated by gain-of-function of *Scr*-HD_{wt} displayed a patch of red pigmentation, an indication that a group of cells might have been transformed to ectopic eye-cells (Fig. 1*G*). Moreover, the presence of one to two sex comb teeth on the ectopic tarsi mediated by Scr-HD_{wt} (Fig. 1*J*) and Scr-HD_{AA} (Fig. 1*K*) indicated that the ectopic tarsi are prothoracic (T1), normally specified by *Scr* (28).

Synthetic Scr Peptides Participate in Protein-Protein Interactions with Pax Transcription Factors in Vivo. At the molecular level, Hox transcription factors are known to participate in protein-protein interactions. Such interactions have been demonstrated to take place between two HDs or a HD and a PAIRED domain (PD), resulting in mutual inhibition of DNA-binding properties, which leads to defects that resemble mutant phenotypes, caused by absence of either gene product. A phenotypic manifestation of such a negative posttranslational regulatory inhibition is the eye reduction caused by ectopic expression of various Hox proteins in the eye-antennal disc, shown to be triggered by binding of the HD of Hox proteins to the PD of eyeless (ey) (30, 31). In accordance with these findings ectopic expression of *Scr*-HD_{wt} and *Scr*-HD_{AA} using dpp^{blink}-Gal4 exhibited strong eye phenotypes (Fig. 2*A–D*) as compared to the full-length peptide (30), ranging from reduction to the complete absence of eyes. No abnormal eye phenotype

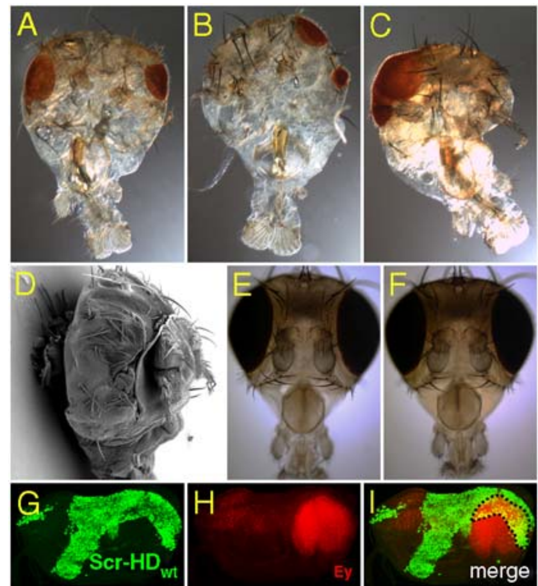

Fig. 2. Synthetic Scr peptides interact with Pax transcription factors in vivo (*A–D*). Eye-reduction phenotypes exhibited by ectopic expression of *Scr*-HD_{wt} (*A*, *B*, and *D*) and *Scr*-HD_{AA} (*C*). According to the strength of expression, different lines exhibited phenotypes ranging from eye-reduction (*A–C*) to eye-absence (*D*). (*E*) Ectopic expression of *Scr*-HD_{DD} resulted in no detectable phenotype. (*F*) Wild type head. (*G–I*) Ectopic expression of *Scr*-HD_{wt} in the eye-disc (*G*) does not repress *ey* (*H*). Dashed lines show the domain of colocalization of Scr-HD_{wt} and Ey (*I*). The dpp^{blink}-Gal4 driver has been used throughout.

was observed with *Scr*-HD_{DD} (Fig. 2*E*), and the eyes in this case were indistinguishable from the control (Fig. 2*F*). Colocalization of Scr-HD_{wt} (Fig. 2*G*) with endogenous Ey (Fig. 2*H*) suggested their interaction at the posttranslational level in the region of coexpression (Fig. 2*I*, *Dashed Line*).

Synthetic Scr-HD Peptides Act as Transcriptional Activators and Repressors in the Eye-Antennal Disc. If Scr-HD peptides can cause antenna-to-tarsus transformations, genes responsible for antennal development must be repressed and leg determination genes must be activated in the antennal disc (32). Ectopic expression of *Antp* or *Scr* in the antennal disc is able to repress *Spalt major* (*Salm*) at the transcriptional level, thus preventing the differentiation to an antenna and allowing the initiation of the leg determination program (21, 33). In agreement with this principle, both Scr-HD_{wt} (Fig. 3*A* and *D*) and Scr-HD_{AA} (Fig. 3*B* and *E*) repressed *Salm*. As expected, no repression was observed with Scr-HD_{DD} (Fig. 3*C* and *F*).

The same paradigm was found to apply for *distal antenna* (*dan*), another antenna determination gene normally expressed in the eye-antennal but not in the leg disc (34). Ectopic *Antp* in the antennal portion of the disc, induced in a mutant *spineless* (*ss*) background, results in the repression of *dan*, thus transforming the antenna into leg structures, a result suggesting that *dan* is repressed in discs undergoing tarsal transformations (34). Our findings show that this also occurs with *Scr*. Scr-HD_{wt} partially repressed *dan* (Fig. 3*G*, arrowhead, and *J*). In the case of Scr-HD_{AA} repression of *dan* was complete (Fig. 3*H* and *K*), while no repression was detected with Scr-HD_{DD} (Fig. 3*I* and *L*).

Similar behavior was expected to apply in the repression of *Homothorax* (*Hth*), the function of which in the antennal determination program has been described extensively (35). Although expressed both in the antennal and the leg disc during development, its expression domain in the antennal disc largely overlaps with *Dll*, whereas in the leg disc the two gene products are distributed to distinct, nonoverlapping regions (36, 37).

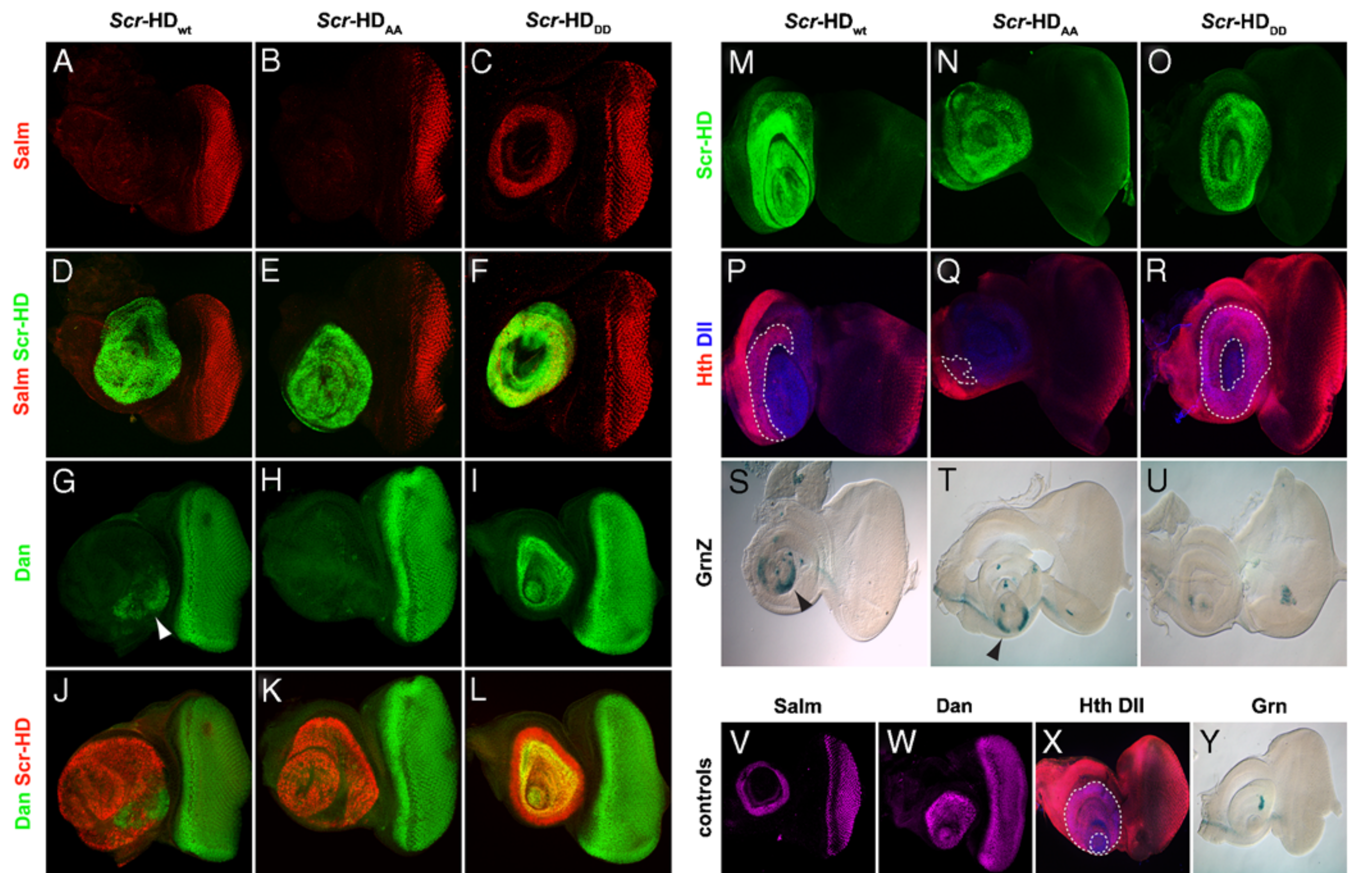

Fig. 3. Synthetic Scr-HD peptides act as transcriptional activators and repressors in the antennal primordium. (*A–F*) Repression of *Spalt major* (*Salm*) in the antennal disc by ectopic expression of *Scr*-HD_{wt} (*A* and *D*) and *Scr*-HD_{AA} (*B* and *E*). No repression was observed with *Scr*-HD_{DD} (*C* and *F*). (*G–L*) Repression of *distal antenna* (*dan*) in the antennal disc is complete with Scr-HD_{AA} (*H* and *K*) and incomplete with Scr-HD_{wt} (*G* and *J*), leaving a patch of cells that retain Dan activity (*Arrowhead* in *G*). These cells do not express ectopic *Scr*-HD_{wt}. (*I* and *L*) Scr-HD_{DD} does not repress *dan*. (*M–R*) Repression of *Hth* results in a shift of the Hth-Dll boundary. Partial repression of *Hth* by Scr-HD_{wt} (*M* and *P*) and Scr-HD_{AA} (*N* and *Q*), as compared to Scr-HD_{DD} (*O* and *R*), where no repression is observed. (*S–U*) X-gal stainings showing the activation of *grain* (*grn*) by Scr-HD_{wt} (*S*) and Scr-HD_{AA} (*T*) (*Arrowheads*). Scr-HD_{DD} (*U*) fails to activate ectopic *grn* expression. (*V–Y*) Normal expression of *Salm* (*V*), *dan* (*W*), *Hth-Dll* (*X*), and *grn* (*Y*) in eye-antennal discs. *Dll*-Gal4 was used to drive expression of all constructs (*A-Y*).

Because ectopic Antp in the antennal disc represses *Hth* (36) we assumed a general mechanism, according to which tarsal transformations in the antenna repress and thus restrict *Hth* outside of the *Dll* expression domain, resulting in a shift of the Hth-Dll boundary. Indeed, ectopic expression of *Scr*-HD_{wt} (Fig. 3*M* and *P*) and *Scr*-HD_{AA} (Fig. 3*N* and *Q*) resulted in partial or complete repression of *Hth* respectively, while no repression was seen with the inactive construct *Scr*-HD_{DD} (Fig. 3*O* and *R*).

Inversely, to probe the transformed antennal discs for ectopically activated leg-specific genes, we tested the synthetic Scr-HD variants in their ability to activate *grain* (*grn*). *grn* encodes a GATA transcription factor (GATAc) that plays an important role in cell rearrangement during morphogenesis. It is expressed in the Central Nervous System (CNS), midgut, and lateral ectoderm during development (38). *grn* is normally expressed in the leg disc but not in the antennal disc; however, it is activated in the antennal disc upon ectopic expression of *Antp* [*grn* enhancer trap lines have been published as rK781 in (33) and as klecks in (39)]. Fig. 3*S–U* show the ectopic activation of *grn* mediated by the wild type and active constructs, whereas Scr-HD_{DD} failed to activate transcription of the reporter (Fig. 3*U*). Taken together, these results demonstrate that the synthetic Scr peptides participate, directly or indirectly, in both transcriptional repression and activation in vivo.

Cells that Maintain *dan* Activity in the Antennal Primordium Give Rise to Ectopic Eyes on the Antennal Tarsi. We were next interested to provide an explanation as to whether the pigmented cells observed in the transformed antennae (Fig. 1*B* and *C*) are indeed eye structures. We hypothesized that cells that retained *dan* activity in the antennal disc (Fig. 3*G*, *Arrowhead*) might account for the formation of ectopic eyes on the transformed tarsi upon misexpression of *Scr*-HD_{wt}. Therefore, we tested the presence of photoreceptor cell markers in Dan protein positive cells in the antennal disc (Fig. 4*A–D*). Ectopic expression of *Embryonic lethal abnormal visual system* (*Elav*) colocalized with cells that still expressed *dan* but not ectopic *Scr*-HD_{wt} (Fig. 4*D*), suggesting that these cells have acquired neuronal fate, similar to the differentiated photoreceptors in the eye portion of the disc. *dan* participates in both antennal and eye development (34, 40), and because these cells cannot differentiate into an antenna, they are able to form ectopic eyes in the transformed A3 segment. Scanning Electron Microscopy (SEM) of adult antennae confirmed that the pigmented cells observed previously (Fig. 1*B* and *C*) are indeed compound eyes, comprising several ommatidia (Fig. 4*E*).

Molecular Interactions of Synthetic Scr Peptides in Live Salivary Gland Cells Using High-Resolution Quantitative APD Imaging. In order to understand at the molecular level differences between the active and inactive peptide variants in their ability to interact with nuclear DNA, we studied their interactions with chromosomal DNA in live cells using advanced fluorescence imaging with Avalanche Photo Detectors (APD imaging) (41). Here we substantiate the phenotypic findings by molecular imaging. Elaborate quantitative study of Scr-HD–DNA interactions in live salivary gland cells is presented in the following paper (42).

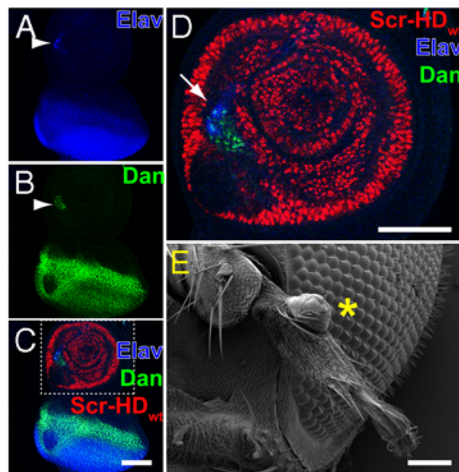

Fig. 4. Cells with no ectopic Scr-HD_{wt} activity fail to repress *dan* but activate *Elav* and thus differentiate into compound eyes. (*A*) *Elav* gain-of-function, (*B*) Dan (*Arrowheads*), and (*C*) merge of (*A*) and (*B*) with Scr-HD_{wt}. (*D*) Magnification of outlined area in (*C*). Arrow points at cells that express both *dan* and *Elav* but not ectopic *Scr*-HD_{wt}. (*E*) Adult antenna transformed into a tarsus, which bears a small ectopic eye in the A3. Note the presence of ommatidia and interommatidial bristles (*Red Asterisk*). Scale bars in (*C*) 100 μm and in (*D*, *E*) 50 μm.

To visualize chromatin, a ubiquitously expressed *histone-H2B-mRFP1* line was used. *mCitrine-Scr-HD* fusions expressed in salivary gland polytene nuclei showed that Scr-HD_{wt} and Scr-HD_{AA} peptides associate significantly with the DNA (Fig. 5). As observed for Scr-HD_{wt} and Scr-HD_{AA}, the transcription factor did not associate uniformly with the DNA but accumulated at sites of loose chromatin conformation where the histone signal was almost absent (Fig. 5*A*, first and second column). These sites should correspond to transcriptionally active regions. In contrast, Scr-HD_{DD} showed some association with chromatin, but the transcription factor was also observed in the nucleoplasm among polytene chromosomes (Fig. 5*A*, third column). To understand this weaker yet substantial association of Scr-HD_{DD} with chromatin we additionally mutated residues 50 and 51 of the Scr-HD to alanines (*Scr*-$HD_{DD}^{Q50AN51A}$). Glutamine-50 and asparagine-51 participate in third helix binding of the Antp HD to the DNA (43). We assumed that these mutations would behave accordingly in the synthetic Scr-HD_{DD} peptide, acting in synergy with T6D and S7D and rendering the peptide interactions with chromatin even weaker. As expected, the synthetic Scr-$HD_{DD}^{Q50AN51A}$ peptides appeared markedly dispersed in the nucleoplasm (Fig. 5*A*, fourth column) and excluded from chromatin. Flies expressing *Scr*-$HD_{DD}^{Q50AN51A}$ constitutively in the embryo, or ectopically in the antennal disc, did not display any of the embryonic or adult phenotypes.

Synthetic Scr-HD Peptides Bind to DNA Specifically in Vitro. To further investigate the observed association of Scr-HD_{DD} with chromatin, we studied the specific binding of the synthetic peptides to DNA in vitro by gel-shift assays (Fig. 5*B*). Using the native Scr binding site *fkh250* and the generic HD-binding site *BS2*, we observed specific binding for both Scr-HD_{wt} and Scr-HD_{AA} but not for the inactive peptides Scr-HD_{DD} and Scr-$HD_{DD}^{Q50AN51A}$, not even at high peptide concentrations (Fig. 5*B*, *Left*). Binding to *BS2* appeared to be stronger for the transcriptionally active variants (Fig. 5*B*, *Right*) and was used for the titration. This result suggested that binding of Scr-HD_{DD} to chromosomal DNA in polytene nuclei is largely nonspecific, because Scr-HD_{DD} has additionally no homeotic function in the fly. Thus, the imaging analysis in live cells and the in vitro binding assay support our genetic findings and strengthen the notion that synthetic *Scr* genes function in a predictable way in vivo.

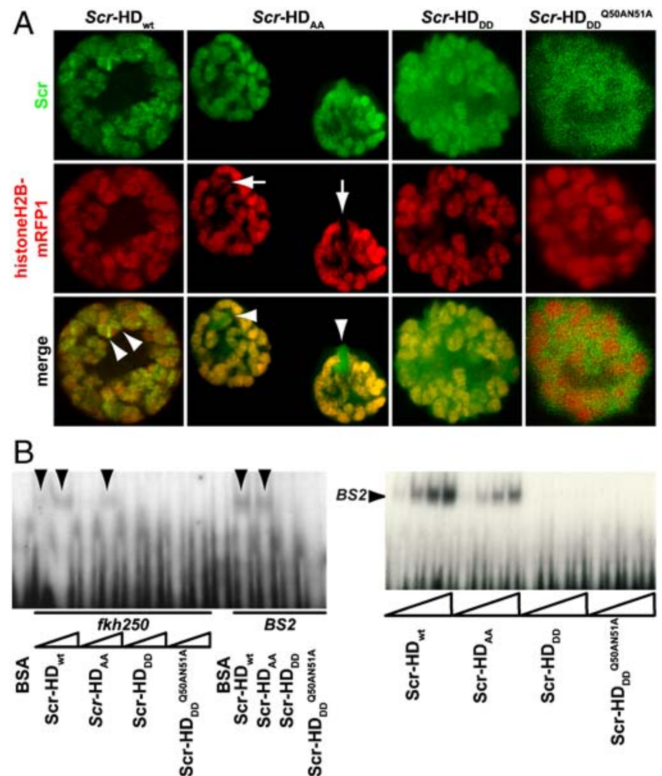

Fig. 5. High-resolution APD imaging of DNA-Scr-HD interactions in live cells. (*A*) Third instar salivary gland polytene nuclei expressing *Scr*-HD_{wt}, *Scr*-HD_{AA}, *Scr*-HD_{DD}, and *Scr*-$HD_{DD}^{Q50AN51A}$ under the control of dpp^{blink}-Gal4. Ubiquitously expressed mRFP1-tagged histone H2B was used to visualize chromatin. Scr-HD_{wt} and Scr-HD_{AA} readily associate with the chromosomes (as shown in the *Green Channel*) but also show sites of accumulation along the chromosome where loose chromatin compaction is shown as a low histone signal (*Arrows*). Arrowheads point at sites of high accumulation observed for Scr-HD_{wt} and Scr-HD_{AA}. The nucleus expressing the inactive *Scr*-HD_{DD} shows some association of the transcription factor with the DNA, but it is also dispersed in the nucleoplasm. There is no pronounced banding pattern observed in this case, which suggests absence of specific binding. Scr-$HD_{DD}^{Q50AN51A}$ appears almost completely excluded from the chromosomes, mainly residing in the nucleoplasm. Scale bars in all cases are 20 μm. (*B*) Electrophoretic Mobility Shift Assay (EMSA) shows that only Scr-HD_{wt} and Scr-HD_{AA} bind DNA specifically in vitro. Both variants bound more strongly to *BS2* than *fkh250* (*Left*). Titration of peptide concentration (*Right*) revealed that even at high concentrations of transcription factor, Scr-HD_{DD} and Scr-$HD_{DD}^{Q50AN51A}$ do not bind DNA (*BS2*) specifically.

Discussion

The genetic role of *Hox* genes has been studied extensively by gain- and loss-of-function experiments (24, 27, 35, 44–46), and the properties of the HD-DNA complex have been elucidated in solution by NMR (43). Nevertheless, the precise mechanisms that orchestrate the transcriptional regulation of the vast number of *Hox* target genes remain elusive. Here we have derived synthetic *Scr* genes and analyzed their function throughout development. We demonstrated that they are able to induce homeotic transformations in the adult fly and embryo, repress and activate antenna and leg-specific genes, respectively, and participate in protein-protein interactions. Our results support that they are functional in vivo and thus question the role of the N terminus of Scr in transcriptional specificity.

Hox-mediated antenna-to-tarsus transformations proceed through repression of genes necessary for antennal specification and ectopic activation of leg-specific genes in the antennal disc.

This transformation is DNA-binding-dependent for Antp (30), because residues that impair binding of the HD to the minor groove (47) also abolish its transformation capacity in vivo. This suggested a similar requirement for Scr and thus for synthetic Scr peptides. Indeed, the latter bound putative Scr and generic HD-binding sites in vitro (Fig. 4*B*), repressed antennal-specific genes (*Salm*, *dan*, and *Hth*) and activated leg-specific genes (*grn*) in the antennal disc (Fig. 3). The inability of Scr-HD_{DD} to trigger any of these phenotypes is in line with substitutions of threonine 6 and serine 7 to aspartates in the full-length protein, which impaired its DNA-binding activity in vitro (19). Our findings suggest that these mutations also abolish the capacity of Scr-HD_{DD} to participate in HD-PD or HD-HD interactions. Although mutation of glutamate 19 to glycine abolished the dimerization capacity of Antp in vivo (30), in the case of Scr other residues of the HD, in addition to glutamate 19, might participate in protein-protein interactions, or, alternatively, the negative charge introduced by the aspartates might be responsible for abolishing these interactions through electrostatic repulsion. The fact that Scr-HD_{AA} exhibited similar phenotypes as Scr-HD_{wt} (compare Fig. 2*C* to Fig. 2*A*, *B* and *D*) is in favor of this scenario.

Surprisingly, the homeotic transformations observed in the adult fly (Fig. 1*E*–*I*) were considerably stronger than the ones observed with the full-length protein (Fig. 6*A* and *C*). Partial antenna-to-tarsus transformations were obtained with both the dpp^{blink} (Fig. 6*A*, as compared to Fig. 4*E*) and the *Dll* enhancers (Fig. 6*C* and *D*, as compared to Fig. 1*E*) and the eye-reduction phenotypes were comparable in strength to the full-length protein (compare Fig. 6*B* to Fig. 2*A*, *B*, and *D*). The same applied in the repression of antennal-specific markers (*Salm* in Fig. 6*E* and *F*, *dan* in Fig. 6*G* and *H*, *Dll* in Fig. 6*I* and *J*, and *Hth* in Fig. 6*K* and *L*) and the greater overlap observed between Hth and Dll (Fig. 6*M*, areas indicated by white dashed lines, as compared to Fig. 3*P*) supported the partiality of the tarsal transformation. Finally, ectopic expression of *Elav* on the antennal disc was found to colocalize with Dan protein positive cells (Fig. 6*N*–*P*). Weaker transformations obtained by the full-length Scr (27, 28) support the notion that the synthetic genes exhibit stronger homeotic function in vivo. In the paradigm of salivary gland induction, a morphogenetic process initiated by *Scr*, the synthetic genes behaved in a predictable way. They triggered the formation of an additional pair of salivary glands in the region of the embryonic head (Fig. 1*A*), comparable to those induced by the full-length protein (19).

Four lines of evidence support that the function of the synthetic genes is specific rather than a generic HD effect. First, the sex comb teeth observed on the antennal tarsi indicate prothoracic leg identity (T1), specified by *Scr*. Second, the ectopic salivary glands in the embryo imply *Scr*-specific function (26). Third, mutations in the HD of the full-length peptide (19) behaved accordingly in synthetic peptides. Finally, specificity of Antp and Scr is owed to the N-terminal part of the HD (44), also present in the synthetic peptides.

It has been proposed that the large N-terminal part of Ubx and Scr is required for transcriptional activation in vivo (48). Our data show that this need not be the case for several *Scr*-specific functions. So, if *Scr*-specificity is achieved by synthetic genes, what is the function of the large N-terminal sequence of Scr in vivo? Sequence comparison of the fly Scr with its insect and vertebrate homologs (Fig. S1) revealed the divergence of the N terminus of the protein in length and sequence, with the exception of the MSSYFVNS, the DYTQL and the SCKYA motifs. Both the fly (48) and the murine MSSYFVNS domain in flies (49) seem to be limited to contributing to transcriptional potency rather than specificity, because its deletion resulted in homeotic transformations (49) and ectopic activation of *Scr* target genes, albeit weaker than the wild type protein (48, 49). Substitution of serine-10 by leucine in the Scr^{14} hypomorphic allele only re-

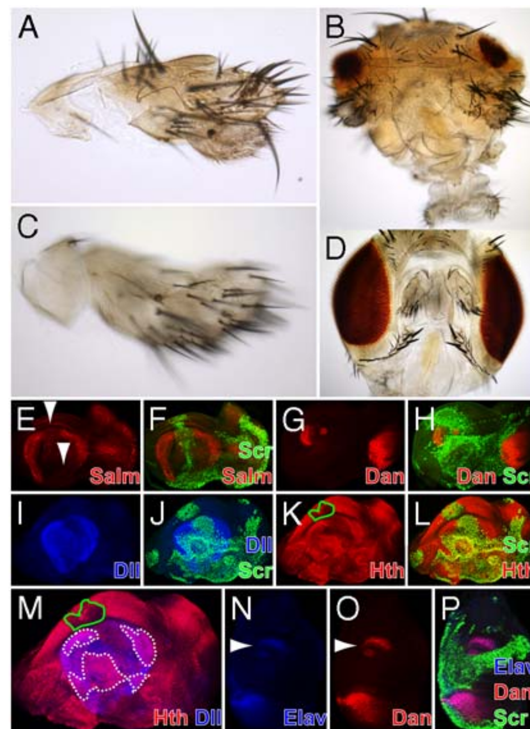

Fig. 6. The full-length Scr peptide exhibits weaker homeotic function than its synthetic counterparts in vivo. (*A*) Partial antenna-to-tarsus transformation mediated by Scr full-length using the dpp^{blink} enhancer. (*B*) The same gain-of-function results in eye-reduction in the head. (*C*) Transformation using the *Dll* enhancer is also incomplete. (*D*) Adult head of a fly expressing Scr full-length using *Dll*-Gal4. (*E*–*F*) Repression of *Salm* by the full-length Scr in the antennal disc (*Arrowheads* in *E*) is complete. (*G*–*H*) The same applies in the repression of *dan*. (*I*–*M*) Repression of *Dll* and *Hth* is incomplete (*I*, *K*, and *M*) and considerable overlap of Hth and Dll is observed in the antennal disc (*Dashed White Lines* in *M*). Scr represses *Hth* also outside the *Dll* expression domain (*Solid Green Line* in *K* and *M*). (*N*–*P*) Cells that maintain Dan activity (*Arrowhead* in *O*) in the antennal disc express ectopic *Elav* (*Arrowhead* in *N*). The full-length *Scr* has been induced by dpp^{blink}-Gal4 (*E*–*P*).

sulted in a mild decrease in the number of sex comb teeth in the adult (50). The same applies to the DYTQL motif, because Scr^{15}, another hypomorphic allele of *Scr* lacking this motif, displayed partially compromised *Scr*-activity but no loss of specificity in vivo (50). When parts of the N terminus of the murine Hox-a5 were removed, its transcriptional activity in live cells and its DNA-binding in vitro were considerably weaker, though not completely abolished (49). Although Hox-a5 lacking the complete N terminus could not trigger homeotic transformations in the fly (49), this indicates that the N terminus is responsible for regulating transcriptional levels. Our results show that the HD, YPWM motif and C terminus of Scr are both necessary and sufficient for providing transcriptional specificity in vivo. A change in potency, rather than in specificity, was also observed for Antp lacking fractions of its N terminus (44), suggesting a general requirement of the latter for transcriptional "fine-tuning" rather than specificity among *Hox* paralogs and homologs. An example of a dose-dependent developmental output of *Hox* gene products has already been described for Ubx-mediated repression of *Dll* (48) and might apply to a few Hox peptides. Quantitative studies might help to precisely describe such features of Hox peptides in vivo.

Our work also supports the hypothesis that synthetic genes and/or peptides behave to a large extent in a predictable way. This notion is central to synthetic biology (51–53), not only for engineering artificial processes, but also for developing new techniques for gene and peptide therapy. In the long run, functional synthetic genes/peptides may bear advantages for medical

applications. Due to their size, they are expected to be considerably easier to engineer/synthesize and have better penetration efficiency into target cells or tissue—one of the greatest obstacles in peptide therapy. More studies in this direction will help unravel such perspectives. In the following paper (42), we take first steps in studying quantitatively the interactions of synthetic Scr peptides with DNA in live cells.

Materials and Methods

Cloning Procedure and Fly Transgenesis. Generation of plasmids was performed using standard procedures. Transgenic lines were generated as described (54). Fly stocks used are outlined in *SI Materials and Methods*.

Immunohistochemistry. Antibody stainings were performed as outlined in *SI Materials and Methods*.

High-Resolution APD Imaging. High-resolution APD imaging was performed on a uniquely modified ConfoCor3 instrument (Carl Zeiss, Jena, Germany) (refer to *SI Materials and Methods* for more detail).

Electrophoretic Mobility Shift Assay (EMSA). All *mCitrine-Scr-HD* fragments were cloned to pCR2.1-TOPO Vector (Invitrogen) and the peptides produced in vitro using TNT® T7 Quick Coupled Transcription/Translation System (Promega). EMSA was then performed as described (31).

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