Plasticity of Drosophila Stat DNA binding shows an evolutionary basis for Stat transcription factor preferences

María Luisa Rivas¹, Laura Cobreros¹, Martin P. Zeidler ² & James Castelli-Gair Hombria¹+

¹Centro Andaluz de Biología del Desarrollo (CABD), CSIC/UPO, Universidad Pablo de Olavide, Seville, Spain, and ²Department of Biomedical Science, MRC Centre for Developmental & Biomedical Genetics, University of Sheffield, Sheffield, UK

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In vertebrates, seven signal transducer and activator of transcription (STAT) proteins bind to palindromic sites separated by spacers of two or three nucleotides (STAT1), four nucleotides (STAT6) or three nucleotides (STAT2 to STAT5a/b). This diversity of binding sites provides specificity to counter semiredundancy and was thought to be a recent evolutionary acquisition. Here, we examine the natural DNA-binding sites of the single Drosophila Stat and show that this is not the case. Rather, Drosophila Stat92E is able to bind to and activate target gene expression through both 3n and 4n spaced sites. Our experiments indicate that Stat92E has a higher binding affinity for 3n sites than for 4n sites and suggest that the levels of target gene expression can be modulated by insertion and/or deletion of single bases. Our results indicate that the ancestral Stat protein had the capacity to bind to 3n and 4n sites and that specific Stat binding preferences evolved with the radiation of the vertebrate Stat family.

Keywords: STAT; binding sites; Drosophila; evolution

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INTRODUCTION

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway was originally discovered in vertebrates on the basis of its transduction of activation of γ-interferon (Darnell, 1997; Levy & Darnell, 2002). Analysis of the pathway rapidly identified a family of seven closely related STATs as well as many pathway-activating cytokines, several receptors and four JAK kinases. The seven STAT transcription factors share several features including an SH2 domain, an invariant tyrosine residue phosphorylated as a result of the activation of STAT, and a characteristic DNA-binding domain. According to the established canonical model, activation of the JAK/STAT pathway by cytokine signalling brings about tyrosine phosphorylation of cytoplasmic STATs and leads to their dimerization. This complex translocates to the nucleus where it binds to DNA, thus activating target gene transcription (Kisseleva et al, 2002; Levy & Darnell, 2002). STAT DNA-binding sites, also known as Gamma interferon activation site (GAS) elements, consist of an essential core comprising the palindromic sequence TTC(n)GAA where n represents a spacer of 2–4 nucleotides. STAT6 shows a preference for 4n spacing, whereas other STATs preferentially bind to 3n, although they can also bind to 2n sites with low affinity (Ehret et al, 2001).

The Jak/Stat pathways identified in invertebrates seem to be much simpler. Of these, only Drosophila has a ‘complete’ pathway comprising three unpaired-like cytokines (Upd, Upd2 and Upd3); one receptor (Domeless, Dome); one JAK kinase (Hop) and one Stat (Stat92E; Binari & Perrimon, 1994; Hou et al, 1996; Yan et al, 1996; Harrison et al, 1998; Brown et al, 2001; Chen et al, 2002; Agaisse et al, 2003; Hombria et al, 2005; reviewed in Hombria & Brown, 2002; Arbouzova et al, 2006).

In vitro site selection and electrophoretic mobility shift assays (EMSAs) showed that Drosophila Stat has a binding preference for sites with 3n spacing (Yan et al, 1996). In vivo, Stat92E binding of 3n sites was confirmed for the even skipped (eve) gene (Small et al, 1996). In cell culture, it was also shown that Drosophilaraf (Draf) and Suppressor of cytokine signalling at 36E (Socs36E), enhancers containing 3n sites are activated by Jak/Stat (Kwon et al, 2000; Baeg et al, 2005; Müller et al, 2005). Furthermore, vertebrate 3n GAS elements act as reporters for the activation of Stat in Drosophila melanogaster and in Caenorhabditis elegans (Gilbert et al, 2005; Wang & Levy, 2006).

These data suggest that the ancestral Stat bound to 3n sites and that the preference for sites with other spacing evolved after
Fig 1 | Stat regulates \textit{dome} transcription through 4n sites. (A) Schematic representation of the \textit{dome} gene showing the localization of the putative Stat-binding sites. Sites in the first intron are represented by black (3n) or red (4n) asterisks. (B) Comparison of the conserved SLF4 region in several Drosophilidae. The element has two putative Stat sites with varying spacer lengths as indicated by the tree branch colours (4n4n (red), 4n3n (orange) and 2n3n (green)). Asterisks under the sequence label the putative Stat-binding sites and red dashes indicate the mutated bases in the SLF2\text{*MUT} and SLF4\text{*MUT}. (C) The SB first intron fragment containing the 3n sites does not drive expression in the embryo. The SL (D) and the SLF2 (E) fragments drive expression in the pharynx and hindgut. (F) Simultaneous mutation of the conserved third and fourth Stat-binding sites in SLF2\text{*MUT} abolishes most expression from the pharynx and hindgut, although low levels remain at late stages. (G) The SLF4 fragment drives expression, albeit at low levels, in the pharynx and hindgut. Hindgut expression is only observed in inserts with higher levels of expression (compare G with Fig 2C). (H) Mutation of both Stat-binding sites abolishes pharynx and hindgut expression in SLF4\text{*MUT}. This particular line has been chosen as it has Jak/Stat-independent expression in the amnioserosa (white arrow) that acts as an internal control for staining. Mutation of only one conserved Stat site in SLF2, either the third site (I) or the fourth site (J), is not sufficient to abolish the expression (compare wild-type SLF2 in (E) with (I) and (J) with the double mutant in (F)). The tree in (B) is modified from the assembly, alignment and annotation of 12 species as published in FlyBase. Stat, signal transducer and activator of transcription.

the vertebrate STAT radiation. Here, we present evidence that the converse is true, with binding site plasticity of STAT transcription factors representing an ancestral state. We show that Drosophila Stat is able to activate transcription through 4n sites. We show that Stat binds to 3n sites with higher affinity \textit{in vitro}, and that the transformation of 4n into 3n sites increases the activation of Stat targets both \textit{in vitro} and \textit{in vivo}. These observations clarify how the Stat binding preferences evolved and illustrate an unanticipated plasticity of DNA binding that will help in the definition of direct Stat targets outside the vertebrate lineage.

RESULTS AND DISCUSSION

Stat92E activates \textit{dome} through 4n sites

Udp expressed in the ectoderm of the Drosophila pharynx and hindgut signals to the adjacent mesoderm where it enhances \textit{dome} transcription (Hombria \textit{et al}, 2005). This effect is mediated by a mesoderm-specific enhancer (\textit{dome-MESO}) present in the first intron of the \textit{dome} gene (Hombria \textit{et al}, 2005). To prove that this enhancer is regulated directly by Stat, we searched for potential Stat92E-binding sites [TTCT(G)GAA] in \textit{dome-MESO} (Fig 1A; Yan \textit{et al}, 1996) and identified three such sites at the 3’ end of the reporter. However, a 1.2-kb lacZ construct, \textit{dome-SB}, containing these 3n sites, is unable to drive mesodermal expression (Fig 1C), whereas the complementary 1.6-kb proximal fragment, \textit{dome-SL}, reproduces the \textit{dome-MESO} pattern of expression (Fig 1D). The subdivision of this fragment locates the enhancer within a 746-bp fragment that we named \textit{dome-SLF2} (Fig 1A,E). Although no canonical 3n sites are present in SLF2, five 4n sites are present—a sequence bound in vertebrates by the STAT6 protein (Ehret \textit{et al}, 2001). As previous \textit{in vitro} binding site selection experiments using Stat92E isolated the 3n sites exclusively, we set out to test whether Drosophila Stat92E could bind to 4n sites \textit{in vivo}.

To identify which of the five potential sites drive mesodermal regulation, we compared the first intron sequence of \textit{dome} in several Drosophilidae (Drosophila 12 Genomes Consortium, 2007). The only conserved sequence is a 43 bp element containing the third and fourth \textit{D. melanogaster} Stat 4n sites (Fig 1B). To test the possible function of these two sites, we made an SLF2 construct with the conserved third and fourth 4n sites mutated (SLF2\text{*MUT}), a 137-bp fragment containing only the two 4n sites present within the 43 bp conserved region (SLF4), and an SLF4 construct with these two 4n sites mutated (SLF4\text{*MUT}; Fig 1B).

Analysis of SLF2\text{*MUT} shows that mutation of the third and fourth 4n sites results in the almost complete loss of mesoderm expression (Fig 1F), although low levels of expression were still observed at late embryogenesis. The SLF4 fragment alone is able to drive expression in the mesoderm of the pharynx and hindgut. The expression of SLF4 is more variable than that of SLF2 with some insertions showing expression exclusively in the pharynx (Fig 1G), whereas others showing low levels of general mesoderm expression in addition to
expression in the pharynx and hindgut (Fig 2C). Mutation of the 4n sites in SLF2*MUT is sufficient to ablate all expression in both the pharynx and the hindgut (Fig 1H).

The lower levels of expression of SLF4 relative to those of SLF2 in the pharynx and hindgut and the slight remnant expression observed in the pharynx of SLF2*MUT embryos suggest that some of the non-conserved 4n sites might contribute to dome-MESO expression. We tested whether both of the conserved 4n sites in SLF2 are necessary by independently mutating them (Fig 1J). Mutation of a single site is not sufficient to abolish mesoderm expression, indicating that these sites are redundant in the context of the SLF2 enhancer.

To confirm that SLF2 and SLF4 are responsive to Jak/Stat signalling, we studied their expression in Df(1)os1A mutants that lack all Upd ligands. As expected, SLF2 expression disappeared from the pharynx and hindgut (Fig 2A,B) and the same is true for SLF4 (Fig 2C,D). Conversely, ectopic activation of the pathway by expression of Upd or Upd2 using the ectoderm-specific 69B-Gal4 line activates SLF2 (Fig 2E; data not shown) and SLF4 (Fig 2F) in the mesoderm. This ectopic activation requires the conserved 4n sites as it is not observed in SLF2*MUT (Fig 2G) or SLF4*MUT (Fig 2H).

The dome-MESO enhancer and its derivatives are expressed specifically in the mesoderm, suggesting that Stat is interacting with tissue-specific cofactors. We tested whether dome-MESO could also be activated in the ectoderm if Upd was co-expressed with various mesoderm-specific proteins. We observed ectopic ectoderm expression after co-expression of Upd with Tinman (Tin; Fig 2I,J), but not with Dome2, Bagpipe or Biniou (Fig 2J; data not shown). This suggests that Tin or one of its downstream targets is a STAT cofactor necessary for dome activation in the mesoderm. The requirement for this interaction explains why only the 4n sites in dome-MESO are functional.

### Stat92E binds to 4n and 3n sites with different affinity

To test whether Stat92E can bind to the conserved 4n sites in vitro, oligonucleotides containing these sites (known as 4n Dome3 and 4n Dome4; Fig 3A) were used in EMSAs together with a Stat92E–GFP (green fluorescent protein) fusion protein activated by co-expression of the constitutively active Jak allele HopFurEl (Luo et al, 1995; Karsten et al, 2006). By using the established 3n wild-type consensus (Yan et al, 1996) as a positive control, a strong band is detectable (Fig 3B, arrow) that is supershifted by the addition of the GFP antibody (Fig 3B, arrowhead). The same binding conditions with the 4n Dome3 and 4n Dome4 sites also give clear band shifts that can be supershifted (and possibly stabilized) by anti-GFP (Fig 3B). However, 4n band shifts are considerably weaker than those produced by the 3n control under these in vitro conditions. We next tested whether 4n Dome sites could compete with wild-type radiolabelled 3n sites (3n wild type) for binding to activated Stat92E–GFP. Although unlabelled 3n wild-type-binding sites are strong competitors (Fig 3C, lanes 1–3), a 50-fold excess of mutant 3n-binding sites is not able to compete with labelled 3n wild type (Fig 3C, lane 14). By contrast, unlabelled 4n Dome3 and, to a lesser extent, 4n Dome4 sites can compete with the labelled 3n wild-type probe (Fig 3C, lanes 6 and 9). A Dome3 + 4 probe containing both 4n sites separated by their D. melanogaster spacer (Fig 1B) at 25-fold excess (Fig 3C, lane 12)—a concentration that provides a 50-fold excess of 4n sites produces stronger competition than the equivalent concentration of either 4n site alone, suggesting that two adjacent sites are able to bind Stat92E better than a single binding site in isolation.

To measure in vivo the relative transcriptional activation potential of activated Stat92E–GFP at these sites, we devised a luciferase reporter plasmid containing either four 3n- or 4n-binding sites (Fig 3D; Methods). By using these reporters in an established KC167 cell-based model (Müller et al, 2005), we stimulated cells by
either co-expressing the Upd ligand or the activated Jak HopTuml 
(Luo et al., 1995). Under these conditions, both 3n and 4n reporters 
show significantly increased levels of activity over the unstimu-
lated state, with the 3n reporter around twice as active as the 4n 
reporter (Fig 3E). Finally, we found that the SB sites (Figs 1A,3A) 
can bind to Stat92E in vitro (Fig 3F), underscoring the importance 
of cofactors for the activity of Stat in vivo.

Comparison of the conserved SLF4 region in various 
Drosophilidae (Fig 1B) shows that species closely related to 
D. melanogaster share the 4n spacing of the Stat fourth site, with 
3n sites present in more distantly related drosophilids. In some 
species in which the fourth site is 3n, the third site has a 2n spacer. 
The above experiments suggest that changes in spacer length 
during evolution might modulate the levels of transcription of 
target genes. To test this possibility, we mutated the fourth 4n 
site in SLF4 to a 3n site as observed in the obscura group. 
The resulting enhancer drove higher levels of expression in 
D. melanogaster than did SLF4 (Fig 4A,B), indicated by the 

Fig 3 | Drosophila Stat binds to 3n and 4n sites with various 
affinities. (A) Sequences of the oligonucleotides used in (B,C,F). SB1, SB2 and SB3 
correspond to the potential Stat-binding sites present in the SB fragment (Fig 1A), and Dome3, Dome4 and Dome3+4 correspond to the 
D. melanogaster Stat-binding sites in SLF4 (Fig 1A,B). 3n wild type (WT) corresponds to the Stat92E-binding site consensus (Yan et al., 1996).

(B) EMSA assay using the radiolabelled binding sites indicated and showing binding activities that are detectable after co-transfection of plasmids 
expressing Stat92E–GFP and the constitutively active HopTuml proteins. Specific shifted bands corresponding to DNA:Stat92E–GFP (arrow) and 
DNA:Stat92E–GFP complexes supershifted with anti-GFP (arrowhead) are indicated. (C) EMSA assay using radiolabelled 3n WT to detect activated 
Stat92E–GFP-binding activity. Each lane contains the same quantity of cell extract and labelled 3n WT-binding site and was co-incubated with the 
indicated fold excess of unlabelled competitor sites. (D) Schematic representation of the reporters used in (E) with black boxes representing Stat-
binding sites. Underlined bases represent the core Stat92E-binding sequence. Another thymine residue (bold) was inserted into each binding site 
of 2x2Draf 3n to create the 4n reporter. (E) Firefly luciferase activity in cells transfected with the reporters shown in (D) and co-transfected with 
plasmids expressing either the pathway ligand unpaired or the constitutively active Jak HopTuml. Levels were normalized to a co-transfected 
constitutively expressed Renilla luciferase plasmid and are expressed as fold change over unstimulated state. (F) EMSA assay of unactivated 
and activated Stat92E–GFP binding to radiolabelled WT, SB1, SB2 and SB3 oligonucleotides. EMSA, electrophoretic mobility shift assay; GFP, green 
fluorescent protein; MUT, mutated; STAT, signal transducer and activator of transcription.

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consistent appearance of hindgut expression in all insertions. Further mutation in this enhancer of the third-site to a 2n spacer, as in D. virilis, restored the levels of expression similar to the original SLF4 enhancer (Fig 4C). These results indicate that varying the relative number of 3n compared with 4n sites might control the level of expression of Stat targets during evolution.

The direct Stat target crb is regulated through 4n sites

To determine whether other Drosophila Stat target genes are controlled through 4n sites, we analysed the Stat-dependent crb spiracle-specific enhancer (Fig 5A,B; Lovegrove et al, 2006). Simultaneous mutation of the three putative Stat-binding sites (one 3n and two 4n sites) reduces crb spiracle expression (Fig 5C). Mutation of the 3n site has little effect on the expression of enhancer (Fig 5D), whereas mutation of both 4n sites (Fig 5E) results in levels of expression similar to those obtained after mutation of both the 3n and 4n sites.

Our results highlight the limitations of bioinformatic and in vitro DNA-binding analyses as sole methods for defining transcription-factor-binding sites if they overlook low-affinity binding sites that might be functional in vivo because of tissue-specific cofactors.

The capacity of Drosophila Stat to activate through both 3n and 4n sites suggests that the ancestral Stat protein had the ability to bind to both sites. This capacity has been retained in Drosophila and possibly in other invertebrates. Intriguingly, this suggests that the Drosophila Stat protein dimer has some flexibility in its ability to bind to DNA sites with different spacing. The loss of this flexibility after the radiation of the vertebrate STATs might have provided a flexible system to modulate the distance from the source at which a given Stat target could be activated.

**METHODS**

Constructs and directed mutagenesis. All reporter constructs were generated in phs43lacZ. To create the SB and SL reporters, the dome-MESO 2.8-kb enhancer fragment (Hombria et al, 2005) was subdivided into a distal (SB) EcoRV-BamH1 and a proximal Ncol-EcoRV (SL) fragment. PCR amplification of the SL fragment with the primers TGGAGGGAACCTGGGATGG and ATGTGGGCCCCATCTGGGC generated 746 bp SLF2. Amplification with CGATAGGTAGGGGGAGCC and GTACATCGGCACTTCGGACG created 138 bp SLF4. Amplified fragments were subcloned into pGEMT and from there into phs43lacZ.

The conserved Stat sites in SLF4 and SLF2 were in vitro mutagenized into TTCCGCTGTT, the third site, and AAGCTGCCGA, the fourth site (Fig 1B); using QuikChange (Stratagene; www.stratagene.com) and appropriate PAGE-purified primers to create SLF2*MUT and SLF4*MUT. These sites were mutated independently in SLF2 to create SLF2*MUT3rd and SLF2*MUT4th. The same sites were also mutated to create various spacer variants of SLF4. In SLF4 4n3n, the fourth site was mutated to TTC.TCCGAA where (.) indicates a deleted G. In SLF4 2n3n, apart from this deletion, the third site was mutated to TTC..TGAA where (.) indicates deleted GC.

The wild-type crb43.2 enhancer and the triple mutant Stat-binding-site spiral enhancer have been described earlier (Lovegrove et al, 2006). The mutations in the triple mutant (- - -) were TTTCATGGC (for 3n), TTCTTTCGT (for 5’ 4n) and TTTCA.GGGGT (for 3’ 4n). In the 3n mutated construct (-4n3n), the 3n site was TTTCATGT. In the double 4n mutant construct
(3n–), the sites were transformed to TTCGTGTCTT (5’ 3n) and TTCAGGTGTG (3’ 4n). For each construct, several independent inserts were analysed using anti-β-galactosidase. For SLF4, SLF2*MUT and SLF4*MUT, 10 inserts were studied in each case to confirm that the expression was consistent. All fly strains have been described by Hombria et al (2005). Df(1)os1A is a deletion for all three Upd ligands (Upd, Upd2 and Upd3).

Electrophoretic mobility shift assays. EMSAs to detect DNA binding of Stat92E were undertaken as described by Karsten et al (2006). Double-stranded DNA probes were generated by annealing GAGGAGTTCCGTGAAGAT and GACATTTCGCGCCACC (4n Dome3), GAGACTTCTGAGAATG and GACCATTTCCGGGAAAAA (4n Dome4), GAGAGGTTCCGCGG (wild type) or GGATTTTATTACT (using Klenow polymerase and dNTPs containing either 32P-dCTP (to generate radioactive probes) or unlabelled dNTPs (to generate cold competitors). EMSAs shown in Fig 3B,F used 0.15 pmol of radiolabelled probe per lane and 50 pmol/ml of competitors. EMSAs to detect DNA binding of the p5n-Draf- Stat-binding domain (known as 2x2Draf 3n) were undertaken in Kc 167 cells as described by Müller et al (2006). The recessed ends of annealed oligonucleotide pairs were filled in using Klenow polymerase and dNTPs containing either 32P-dCTP (to generate radioactive probes) or unlabelled dNTPs (to generate cold competitors). Both the original 3n– sites (known as 2x2Draf 4n) and the newly generated 4n– sites were transected to TTCGTGTCTT (5’ 3n) and TTCAGGTGTG (3’ 4n). For each construct, several independent inserts were analysed using anti-β-galactosidase. For SLF4, SLF2*MUT and SLF4*MUT, 10 inserts were studied in each case to confirm that the expression was consistent. All fly strains have been described by Hombria et al (2005). Df(1)os1A is a deletion for all three Upd ligands (Upd, Upd2 and Upd3).

Luciferase assays. The 2x2Draf 3n luciferase reporter is based on the p5’–663 DraWT-luc originally containing two 3n Stat-binding sites (Kwon et al, 2000) that we duplicated to generate a reporter containing four 3n sites. We also generated another version of p5’–663 DraWT-luc in vitro which was mutated to include another spacer nucleotide that we duplicated to generate a reporter containing four 3n sites (known as 2x2Draf 4n).

Transformation of the 3n– to 4n– sites was generated in vitro using the QuikChange method (Stratagene) and the oligonucleotides GGGAGTATCTAAATTCTGCTCGAAGGTATATAAATTTCTGCGAAGGATGTTAAGGGTATAGGCTGAGG and GGAGGATCTTTACATTTGCAACCGGAGG (underlined bases represent the core Stat92E-binding sequence and bold indicates the added base). Both the original 3n– and the newly generated 4n– vectors were then monomerized by cutting out the binding sites using BamHI and XbaI, filling in and re-ligating into the parental vector cut Smal to generate 2x2Draf 3n and 2x2Draf 4n, respectively. Constructs were sequence verified. Activity assays were undertaken in Kc167 cells as described by Müller et al (2005). Equal quantities of both 3n– and 4n– reporters were transfected for each experiment repeated in triplicate and normalized to unstimulated background activity.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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