Expression of v-*src* in mammary epithelial cells induces transcription via STAT3

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Transgenic mouse models of mammary tumorigenesis and analyses of human breast tumour samples have indicated a role for Src proteins in the tumorigenic process. The downstream effectors of Src function in mammary epithelial cells are less well understood. STAT proteins constitute a family of transcription factors whose activation by cytokine and non-cytokine receptors leads to tyrosine phosphorylation, dimerization and translocation from the cytoplasm to the nucleus. In the nucleus they activate the transcription of specific genes by binding to consensus DNA elements. STATs 1 and 3 can be activated by both cytokine and non-cytokine receptors, and bind as homodimers or heterodimers to viral simian sarcoma virus (sis)-inducible elements such as that found in the c-*fos* promoter. Here we report that one of the downstream effectors of Src function in mammary epithelial cells is STAT3. We demonstrate that v-*src* expression in mammary epithelial cells induces Tyr-705 phosphorylation, nuclear translocation and DNA binding of STAT3. Furthermore, we demonstrate that v-*src* can induce STAT3-dependent transcription. These observations are the first direct evidence that v-*src* can regulate transcription through the activation of STAT proteins, and add a further level of complexity to the understanding of the mode of action of v-*src*.

INTRODUCTION

The Src tyrosine kinases have long been established as potential oncogenes. v-*src* and mutated forms of c-*src* are capable of transforming many different cell types. Several lines of evidence point to a role for c-*src* in breast cancer. Tyrosine kinase activity is markedly elevated in breast tumours compared with normal tissue [1]; the cytosolic fraction in particular has been found to contain approx. 14-fold elevated tyrosine kinase activity, of which 70% could be specifically immunoprecipitated by c-*src* antibodies [2]. Transgenic mouse models of mammary tumorigenesis also indicate a role for c-*src*. Polyoma middle T (PymT)-induced mammary tumorigenesis seems to require c-*src* because the tumours and lung metastases possess high levels of Src tyrosine kinase activities, and fail to form when PymT mice are bred into a $src^{-/-}$ background [3]. *Neu*-induced mammary tumours in transgenic mice possess elevated Src tyrosine kinase activity [4]. Finally, expression of mutationally activated c-*src* in the mammary glands of transgenic mice causes developmental abnormalities, hyperplasia and a predisposition to neoplasia [5].

v-*src* is presumed to exert its effects by activating various cellular signalling pathways that require p21 Ras [6]. Recently however, Ras-independent transformation by v-*src* has been noted [7]. v-*src* is known to activate both the mitogen-activated protein (MAP) kinase pathway ([8,9], and P. D. Smith, unpublished work) and the Jnk/Sapk signalling cascade [10]. Other signalling molecules activated by v-*src* include phosphatidylinositol 3-kinase [11] and protein kinase C [12]. v-*src* is known to phosphorylate a variety of proteins; however, the consequences of these modifications for intracellular signalling are, for the most part, unknown.

Signal transducers and activators of transcription (STATs) constitute a family of latent transcription factors whose activation is dependent on tyrosine phosphorylation at a site in their C-termini (Tyr-705 in STAT3) [13]. Tyrosine phosphorylation of STAT proteins induces dimerization, followed by translocation to the nucleus, where they bind to DNA response sequences and thereby regulate gene expression. STATs can be activated by cytokines such as the interferon and interleukin families, in which case the activation is mediated by one or more members of the Janus kinase (JAK) tyrosine kinase family. STATs can also be activated by non-cytokine receptors, the best characterized example is the activation of STATs 1 and 3 by the epidermal growth factor receptor, which does not appear to involve JAK family members [14,15]. Activated STATs 1 and 3 can bind to a response element in the c-*fos* gene, called the sis-inducible element (SIE), either as homodimers or as a heterodimer. When subjected to electrophoretic mobility-shift assay (EMSA), they give a characteristic three-banded shift pattern comprising sis-inducible factor a (SIFa) (STAT3 homodimer), SIFb (STAT1:STAT3 heterodimer) and SIFc (STAT1 homodimer). Four recent papers have demonstrated that Src proteins, v-*src* in particular, can phosphorylate STAT3, resulting in the formation of SIFa complexes, although, critically, none have determined whether the STAT3 that is activated by v-*src* can induce transcription [16–19].

Here we describe that expression of v-*src* in the human mammary epithelial cell line HB4a [20] induces tyrosine phosphorylation of STAT3 at Tyr-705, nuclear translocation and sequence-specific DNA binding to a SIE consensus oligonucleotide, resulting in the formation of a SIFa complex. In addition, we provide the first evidence that v-*src* is able to promote transcription through a promoter containing high-affinity (SIE) binding sites for STAT proteins. This is inhibited by a dominant-negative STAT3 mutant, in which Tyr-705 is replaced by a phenylalanine residue [21]. These results support the conclusion that v-*src* regulates transcription through the activation of STAT3.

Abbreviations used: STAT, signal transducer and activator of transcription; JAK, Janus kinase; sis, simian sarcoma virus; SIE, sis-inducible element; SIF, sis-inducible factor; EMSA, electrophoretic mobility-shift assay; MAP, mitogen-activated protein; CSF-1, colony-stimulating factor 1.

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EXPERIMENTAL

Plasmids

The v-*src* cDNA was inserted into the *Bam*H1 site of pREP8 (Invitrogen). Three copies of M67 SIEwt (AGCTTCATTTCC-CGTAAATCGTCGA) and SIEm (AGCTTCAGTTCACGT-CAATCGTCGA) [22] were inserted into *Sma*1- and *Bgl*II-cut pGL3promoter (Promega) to produce the luciferase reporter vectors pSIEwtluc and pSIEmluc. To control for transfection efficiency pCMVβ-galactosidase (in which CMV stands for cytomegalovirus) (Clontech) was co-transfected in all transcription assays. pSTAT3, pSTAT3Y705F (in pMS1), pFlag-STAT3 and Flag-Y705F (in pTL1) were the gift of Dr. Michael Saunders (Glaxo Wellcome) and have been described elsewhere [21]. An empty vector control plasmid was generated by excising the STAT3 cDNA from pSTAT3 and re-ligating. The pREP8*brk* cytoplasmic tyrosine kinase expression vector has been described previously [23,24].

Cell culture

The mammary cell line HB4a derived as described [20] was cultured in RPMI 1640 medium containing 10% (v/v) foetal bovine serum, hydrocortisone (5 mg/ml), cholera toxin (0.1 mg/ml) and insulin (5 mg/ml).

Luciferase reporter assays

Transfections were performed by using the Lipofectamine reagent (Gibco-BRL). For transient transfections, 2×10^5 cells were plated in each well of a six-well dish and transfected with 5 μl of Lipofectamine reagent and 1 μg of reporter DNA, 1 μg of co-transfected plasmid (v-*src* or STAT3, for example) and 0.5 μg of pCMVβ as a transfection control. Cells were harvested 40–48 h after transfection. Luciferase and β-galactosidase assays were performed with commercially available reagents (Promega).

Immunoprecipitation and Western blotting

Cells (5×10^5) on a 60 mm plate were transfected by using Lipofectamine reagent with either pSTAT3-FLAG or pY705F-FLAG, together with either pREP8 or pREP8v-*src*; 48 h after transfection the cells were lysed as described [21] and immunoprecipitated with anti-FLAG M2 affinity gel (IBI). Western blots of the immunoprecipitated STAT3 were probed either with Y705 phospho-specific STAT3 polyclonal antibody (New England Biolabs) or a STAT3 rabbit polyclonal antibody (Santa Cruz Biotechnology). The bound antibodies were revealed with peroxidase-conjugated second antibodies and SuperSignal Substrate (Pierce).

EMSA

Cells (10^6) were transfected by the Lipofectamine procedure; 48 h after transfection, DNA binding proteins were prepared as described previously [25]. EMSA assays were performed with 2% of the DNA-binding proteins obtained from 10^6 cells and 0.9 pmol of ^{32}P-labelled M67SIEwt oligonucleotide [22] in 4% (v/v) Ficoll/1 mM $MgCl_2$/20 mM Hepes (pH 7.9)/1 mM dithiothreitol/50 mM NaCl/50 mg/ml poly(dI-dC)·poly(dI-dC), with or without a 50-fold molar excess of unlabelled competitor SIE oligonucleotide. Supershifting of the complexes was performed by adding 2 μg of either STAT3- or STAT1-specific antibodies (Santa Cruz) at room temperature for 30 min, after incubation of the nuclear extract and the probe. Samples were electrophoresed on a 4% (w/v) polyacrylamide gel run in 0.25 × Tris/borate/EDTA at 150 V for 3–4 h at 4 °C.

RESULTS

Expression of v-*src* in mammary epithelial cells results in the phosphorylation of Tyr-705 and nuclear translocation of STAT3

FLAG epitope-tagged versions of STAT3 and the dominant-negative STAT3Y705F [21], in which Tyr-705 is replaced by a phenylalanine residue, were used to investigate the ability of v-*src* to phosphorylate STAT3. These were transiently co-transfected with either pREP8 vector control or v-*src*. The epitope-tagged STAT3 was immunoprecipitated with anti-FLAG monoclonal antibody, and a Western blot was probed with a phospho-specific STAT3 antibody that detects phosphotyrosine at residue Tyr-705 on STAT3. Figure 1(A) shows that in growing HB4a cells, STAT3, but not STAT3Y705F, was weakly recognized by the antibody (lanes 1 and 2, lane numbering is from left to right), confirming its specificity. However, in v-*src*-transfected cells, STAT3 was heavily phosphorylated at Y705 (Figure 1A, lane 3); again, the antibody did not recognize STAT3Y705F (Figure 1A, lane 4). Figure 1(B) demonstrates that the results presented in Figure 1(A) cannot be accounted for by the amounts of epitope-tagged STAT3 that had been immunoprecipitated; in fact, considerably less STAT3 was immunoprecipitated from the v-*src*-transfected cells than from cells transfected with the vector control. For transcriptional activity, tyrosine phosphorylation of STAT3 is followed by dimerization

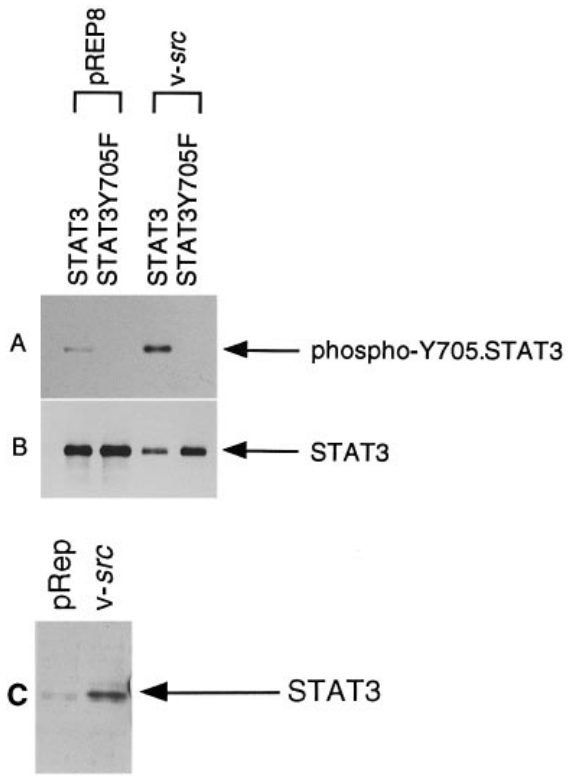

Figure 1 Phosphorylation of Tyr-705 and nuclear translocation of STAT3 in HB4a cells transfected with v-*src*

HB4a mammary epithelial cells were transfected with FLAG epitope-tagged STAT3 and STAT3Y705F, and either pREP8v-*src* or pREP8. Tagged STAT proteins were immunoprecipitated and Western blots were probed with either a Tyr-705 phospho-specific STAT3 antibody (New England Biolabs) (**A**) or a STAT3 antibody (Santa Cruz Biotechnology) (**B**). (**C**) Western blot of nuclear proteins prepared from HB4a cells transfected with STAT3 and either pREP or v-*src*, probed with anti-STAT3 antibody.

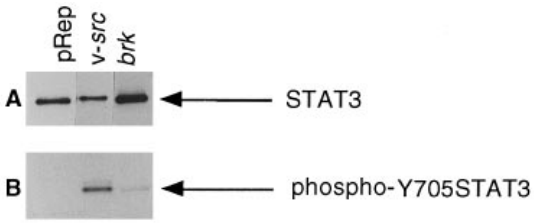

Figure 2 Specificity of Tyr-705 phosphorylation of STAT3 by cytoplasmic tyrosine kinases

HB4a cells were transfected with STAT3 and one of pREP, v-*src* or *brk*. Cell extracts were Western blotted and probed with either a STAT3 antibody (**A**) or a Tyr-705 phospho-specific STAT3 antibody (**B**).

and nuclear translocation. To investigate this, Western blotting was performed on nuclear proteins prepared for EMSA analysis (see Figure 3) from cells transfected with STAT3 and either pREP8 or v-*src*, with an anti-STAT3 antibody. Substantially more STAT3 was detected in nuclear extracts prepared from cells transfected with v-*src* than from cells transfected with pREP8 (Figure 1C).

To investigate the specificity of the phosphorylation of STAT3 by v-*src*, epitope-tagged STAT3 was co-expressed with the *brk* cytoplasmic tyrosine kinase, which is active in HB4a cells [24]. Western blots of immunoprecipitations with anti-FLAG antibody were probed with either STAT3 (Figure 2A) or antibodies against phosphorylated STAT3 (Figure 2B). The results clearly

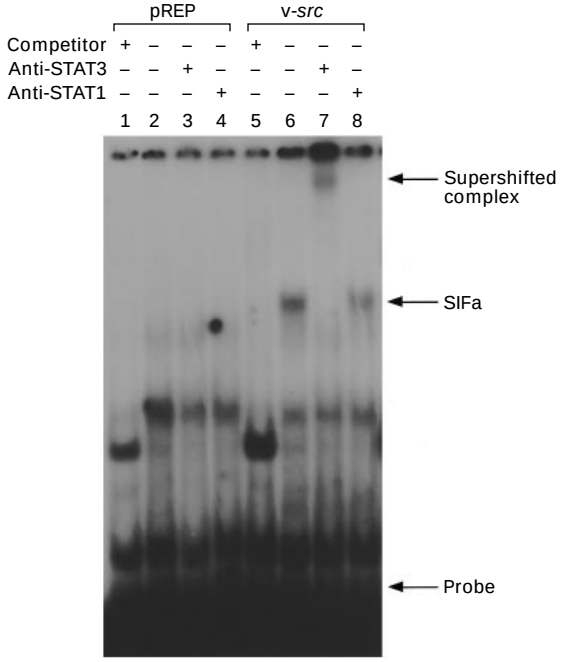

Figure 3 Transient expression of v-*src* and STAT3 in HB4a mammary epithelial cells results in SIF1 complex formation

Nuclear proteins were prepared from HB4a cells transfected with STAT3 and either pREP8 or pREPv-*src*. EMSA was performed with a labelled M67wt SIE probe. The use of excess unlabelled competitor attests to the specificity of the DNA complex formation. The presence of STAT3 in the v-*src*-inducible complex is demonstrated by supershift analysis with either anti-STAT3 or anti-STAT1 antibodies.

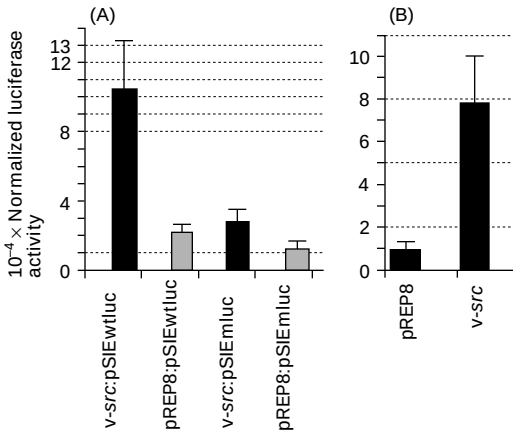

Figure 4 Expression of v-*src* in HB4a mammary epithelial cells induces SIE-dependent transcription

HB4a cells were transfected with either a wild-type (pSIEwtluc) or mutant (pCIEmluc) CIE luciferase reporter construct and either pREP8v-*src* or pREP8 (empty vector control). A β-galactosidase-expressing construct was used to control for transfection efficiency. (**A**) Effects of co-expression of either v-*src* or pREP8 on the activities of the wild-type and mutant SIE reporters; (**B**) SIE-dependent transcription, obtained by subtracting the relative luciferase activity obtained with pSIEmluc from that obtained with pSIEwtluc. Results are means $\pm$ S.E.M. for five independent experiments.

demonstrate that *brk* cytoplasmic tyrosine kinase does not significantly phosphorylate STAT3 at Tyr-705. Consistent with this result, *brk* failed to activate SIE-dependent transcription (results not shown).

Transient expression of v-*src* in mammary epithelial cells induces SIE-dependent DNA-binding activity by STAT3

To confirm that transient expression of v-*src* can promote SIE-dependent DNA binding by STAT3, we performed EMSA on nuclear proteins prepared from HB4a cells transiently transfected with v-*src* and STAT3. Figure 3 shows that SIE DNA-binding activity (SIFa), which can be competed by excess unlabelled wtSIE oligonucleotide, was present in cells co-transfected with STAT3 and v-*src*. The presence of STAT3 and not STAT1 in this complex was confirmed by supershift analysis with antibodies against either STAT3 or STAT1 (the STAT1 antibody is able to supershift SIFc complexes present in nuclear extracts prepared from HB4a cells treated with interferon γ; results not shown). Only the anti-STAT3 antibody was able to supershift the v-*src* inducible complex. In this EMSA the oligonucleotide used was identical with that incorporated into the reporter construct used subsequently.

Expression of v-*src* in mammary epithelial cells activates SIE/STAT3-dependent transcription

To assess whether v-*src* can induce STAT3-dependent transcription, two reporter vectors containing three copies of either a high-affinity SIE site (SIEwtluc) or a mutant SIE demonstrated not to generate SIF complexes (SIEmluc) ([22], and P. D. Smith, unpublished work), cloned upstream of a minimal promoter driving a luciferase gene, were constructed (see the Experimental section). These vectors were co-transfected with either v-*src* or pREP8 vector control, and a plasmid encoding β-galactosidase as a transfection control. Figure 4(A) shows that in the presence of co-expressed v-*src*, the wild-type SIE directed 5-fold more

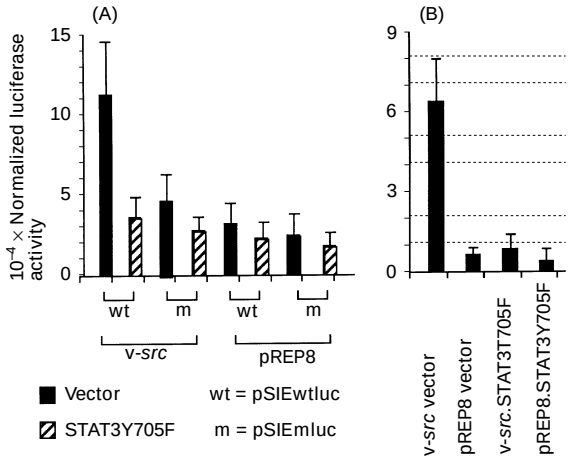

Figure 5 Activation by v-*src* of SIE-dependent transcription is inhibited by co-expression of a dominant negative STAT3

HB4a mammary epithelial cells were transfected with the DNA combinations indicated, and either pSIEwtluc or pSIEmluc plus a β-galactosidase control plasmid. (**A**) Results are means ± S.E.M. for four determinations. (**B**) SIE-dependent transcription (calculated as in Figure 4) induced by v-*src* in the presence or absence (vector) of dominant-negative STAT3Y705F. Results are means ± S.E.M. for four determinations.

promoter activity than that containing mutant SIEs. In pREP8-transfected cells the wild-type SIE containing reporter was twice as active as the mutant reporter. SIE-dependent transcription, obtained by subtracting the relative luciferase activity obtained with SIEmluc from that with SIEwtluc, is shown in Figure 4(B). v-*src* induces 8-fold activation of SIE-dependent transcription compared with the empty vector control.

To determine whether STAT3 is required for the v-*src*-dependent transcription, a vector expressing STAT3Y705F was co-transfected with v-*src*. This mutant has previously been shown to act in a dominant-negative fashion to inhibit STAT3-dependent transcription of an interleukin-6-responsive promoter in HepG2 cells [21]. Figure 5(A) shows that v-*src* induced the wild-type SIE-containing reporter, and that this induction was almost completely abolished by co-expression of STAT3Y705F. Figure 5(B) shows that SIE-dependent transcription induced by v-*src* was almost completely inhibited by co-expression of STAT3Y705F.

DISCUSSION

In this paper we demonstrate that v-*src* expression in mammary epithelial cells leads to phosphorylation of STAT3 at Tyr-705, nuclear translocation and DNA binding of STAT3 homodimers to a SIE consensus oligonucleotide. Further, v-*src* activates STAT3-dependent transcription from a promoter containing SIE sequences. To our knowledge this is the first demonstration of STAT3-dependent transcription induced by an Src tyrosine kinase. Previous publications describe only phosphorylation of STAT3, and DNA binding [16–19]. This is a significant observation because it has been suggested that STATs have evolved primarily to mediate cytokine signalling [13]. Indeed, reports of STAT activation by non-cytokine receptors often omit an analysis of induction of gene transcription [15]. Moreover, at least one study suggests that constitutive STAT1 tyrosine phosphorylation and DNA binding is not sufficient to induce target gene transcription [26]. Investigations into the role of phosphorylation of Ser-727 at the C-termini of both STAT1 and STAT3 have revealed that tyrosine phosphorylation and DNA binding are not dependent on Ser-727 phosphorylation [27,28]. However, activation of transcription is markedly dependent on phosphorylation of Ser-727 [27], particularly in STAT1. Thus it might not be valid to assume that activation of STAT proteins, as assessed by tyrosine phosphorylation and DNA binding, always results in transcriptional regulation.

A question that needs to be clarified is whether c-*src* can also activate STAT3. Yu et al. [16] showed that NIH3T3 cells overexpressing c-*src*, activated Y527Fc-*src*, or v-*src* contain constitutive STAT3 DNA-binding complexes. The level of DNA-binding activity was correlated with transforming capacity; thus c-*src*-expressing cells demonstrated the lowest levels of SIFa complexes and v-*src* the highest. In NIH 3T3 cells that had been transfected to express the colony-stimulating factor 1 (CSF-1) receptor, Cao et al. [17] showed CSF-1-dependent SIFa complex formation; this did not occur in cells expressing a receptor mutant impaired in its ability to both bind Src family members and to induce proliferation in response to CSF-1. Given similar data that platelet-derived growth factor-mediated activation of STAT3 is also correlated with Src association ([17], and references therein), one could propose a general role for Src in integrating growth factor signalling with activation of STAT3.

The dominant-negative STAT3 used in this study was originally described to abolish activation of STAT3 in response to interleukin 6 in HEPG2 cells [21]. The authors suggest mechanisms by which this might be achieved; first by saturating the STAT3-binding site on gp130, and secondly by forming a non-functional heterodimer with activated STAT3. In Src activation of STAT3, the identity of the STAT kinase is unknown. Investigations into the activity of JAK tyrosine kinases in v-*src*-transfected cell lines reveal an elevation of JAK1 activity in mouse fibroblasts [29], but no difference in murine myeloid cells [19]. Because v-*src* has been shown to associate with STAT3 [17,19] the STAT3 kinase is therefore presumed to be Src itself. A most likely model for Src activation of STAT3 would be interaction between the STAT3 SH2 domain and phosphotyrosine residue(s) on Src, followed by tyrosine phosphorylation of STAT3. Evidence of an association between v-*src* and STAT3 *in vivo* [17,19] supports this model. Future studies will involve the use of Src mutants to uncover the key determinants of STAT3 activation by Src. The dominant-negative STAT3 will also be useful in assessing whether the activation of STAT3 is essential for other v-*src*-mediated responses.

Increasing evidence points to a role for serine phosphorylation, possibly achieved via the MAP kinase pathway [27,30–32], in STAT activation. However, interleukin-2-dependent activation of STAT5 apparently requires a serine/threonine kinase pathway other than the MAP kinase pathway [33]. v-*src* is known to activate the c-*jun* N-terminal kinase pathway (JNK) [10] and activates the MAP kinase pathway in HB4a cells (P. D. Smith, unpublished work) and Rat-2 cells [8]. It is therefore of interest to note that a glutathione S-transferase–STAT3 fusion protein was phosphorylated on both tyrosine and serine residues when incubated with lysates prepared from cells expressing v-*src* [17]. The use of dominant inhibitors of these pathways in SIE reporter assays will allow us to determine whether these pathways are involved in the activation of STAT3-dependent transcription by Src.

It is interesting to note recent observations that indicate a role for STAT3 in mammary gland biology. Philp et al. [34] have reported differential activation of STAT3 during murine mammary gland development; specifically, STAT3 is activated in the highly apoptotic involuting gland and during the highly proliferative phase of early pregnancy. Further, Watson and Miller

[35] have demonstrated STAT3 DNA-binding activity in nuclear extracts derived from breast carcinomas. Thus, given that Src is highly active in breast carcinomas, it is tempting to speculate that one consequence of this is the activation of STAT3.

Although v-*src* is implicated in the deregulation of several genes [9,36–38], it is not clear which of them are the key mediators of Src function. Given that we have demonstrated that v-*src* can activate transcription via STAT3, it will be a chief objective of future work to uncover cellular genes whose induction by v-*src* is STAT3-dependent and to analyse how the activation of STAT influences or is influenced by other signalling events on the same promoter(s).

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