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Three loci related to the *src* oncogene and tyrosine-specific protein kinase activity in *Drosophila*

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Rous sarcoma virus (RSV) is an acutely oncogenic avian retrovirus which induces sarcomas in animals and transforms fibroblasts in cell culture. Genetic analysis indicates that the viral *src* gene (v-*src*) mediates neoplastic transformation[1]. The product of v-*src* is a 60,000 molecular weight (MW) phosphoprotein (pp60$^{v\text{-}src}$) possessing the enzymatic activity of a tyrosine-specific protein kinase[2–6]. The viral *src* gene is derived from a cellular gene (c-*src*) which also encodes a 60,000 MW phosphoprotein (pp60$^{c\text{-}src}$) with tyrosine-specific protein kinase activity[4–10]. Both birds and mammals are known to possess c-*src*[7,8]. Shilo and Weinberg have reported that the genome of the fruit fly, *Drosophila melanogaster*, contains nucleotide sequences that are homologous to v-*src*[11]. We report here the molecular cloning and chromosomal mapping of three loci from the *Drosophila* genome that contain such sequences. We also show that *Drosophila* contain both phosphotyrosine and a tyrosine-specific protein kinase activity immunoprecipitated by antisera directed against pp60$^{v\text{-}src}$. It should now be possible to identify the precise locus that encodes a *src*-specific protein kinase in *Drosophila*, and to explore the role of c-*src* in the growth and development of *D. melanogaster*.

A recombinant DNA library of the *Drosophila* genome cloned in bacteriophage λ (ref. 12) was screened by hybridization with a ^{32}P-labelled 800 base pair (bp) *Pvu*II fragment of v-*src* (Fig. 1). Thirty positive clones were isolated and placed in one of three groups on the basis of analysis with restriction endonucleases. All positive clones were isolated several times. It is therefore unlikely that additional sequences that are homologous to v-*src* exist within the *Drosophila* genome. The approximate location of the nucleotide sequences within v-*src* that are homologous to the *Drosophila* clones was determined.

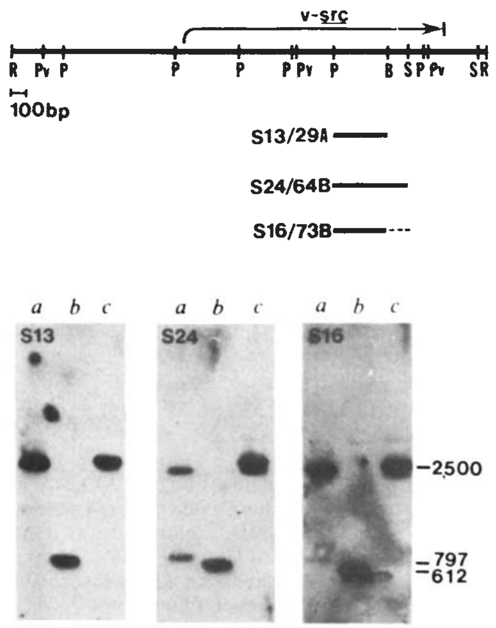

Fig. 1 Location within v-*src* of the sequences that hybridize to *Drosophila* clones S13, S24 and S16. The restriction map of the *Eco*RI-B fragment of the Schmidt–Ruppin A strain of RSV is shown (P = *Pst*I, B = *Bgl*I, S = *Sph*I, Pv = *Pvu*II)[20]. The v-*src* coding sequences are indicated by the arrow. A plasmid (1 μg) containing the *Eco*RI-B fragment was digested with *Eco*RI and *Bgl*I (*a*), *Eco*RI and *Pst*I (*b*) and *Eco*RI and *Sph*I (*c*) and then electrophoresed and blotted onto nitrocellulose by the method of Southern[13]. The blot was probed with the nick-translated ^{32}P-labelled DNA of the *Drosophila* clone indicated on each panel and autoradiographed. Hybridization conditions were as described by Shilo and Weinberg with washing at 55 °C[11]. The approximate sizes of the bands are indicated to the right of the autoradiograms. The extents of homology are summarized under the map of v-*src*. The dotted line indicates weak homology. Similar hybridization conditions were used for probing the *Drosophila* genomic library.

Restriction fragments from a plasmid containing the entire v-*src* gene were separated by electrophoresis through agarose gels, blotted from the gel onto nitrocellulose, and hybridized with ^{32}P-labelled DNA from different *Drosophila* clones[13]. Figure 1 shows the results using a single clone from each of the three groups. The patterns are distinct. Clone S13 hybridizes only with a 400 bp region defined by *Pst*I and *Bgl*I sites. Clone S16 hybridizes strongly to the same 400 bp region and weakly to a 100 bp region defined by *Bgl*I and *Sph*I sites. Clone S24 hybridizes strongly with both the 400 bp and 100 bp regions.

It is interesting that the region of v-*src* with which all three clones are homologous encodes a portion of the enzymatically active domain of pp60$^{v\text{-}src}$ (ref. 14). This region of v-*src* also shows considerable homology to v-*fps*, the oncogene of Fujinami sarcoma virus, and v-*abl*, the oncogene of Abelson murine leukaemia virus (ref. 15 and D. Baltimore, personal communication). Both v-*fps* and v-*abl* are thought to encode tyrosine-specific protein kinases[16–18]. As indicated by Southern blot hybridization, *Drosophila* clones S16 and S13 show homology with v-*fps*, whereas clone S24 does not. Clone S16 also shows homology to v-*abl*, while S13 and S24 do not (data not shown).

Hybridization experiments with clones S13, S16 and S24 show little cross-hybridization among the three clones. This has allowed us to determine the chromosomal location of each clone without interference from related sequences in the other clones. *In situ* hybridization to *Drosophila* salivary chromosomes indicates that S13 maps to region 29A, S24 to region 64B and S16 to region 73B (Fig. 2).

If the genome of *Drosophila* contains genes that are functionally equivalent to *src*, *Drosophila* cells should possess tyrosine-specific protein kinase activity. We tested this

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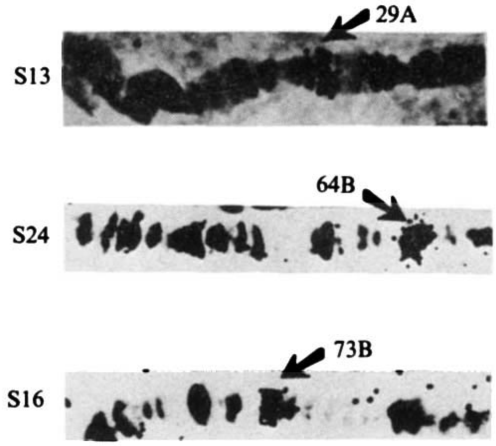

Fig. 2 The chromosomal locations of clones S13, S16 and S24. Salivary glands from third-instar Canton S larvae were squashed as described by Gall and Pardue[21] onto slides that had been pretreated by the method of Brahic and Haase[22]. Squashes were heat-treated and prepared for hybridization as described by Bonner and Pardue[23]. The hybridization solution was 300 mM NaCl, 30 mM sodium citrate, 30 mM sodium phosphate at pH 7.0, 40% formamide, 10% dextran sulphate, 300 μg ml^{-1} sonicated salmon sperm DNA and 1×10^7 c.p.m. ml^{-1} of probe DNA that had been ^{3}H-labelled to a specific activity of 10^7 c.p.m. per μg DNA. Hybridization was for 16 h at 42 °C under siliconized coverslips using 20 μl of hybridization solution per slide. Slides were washed, autoradiographed and stained as described by Gall and Pardue[21].

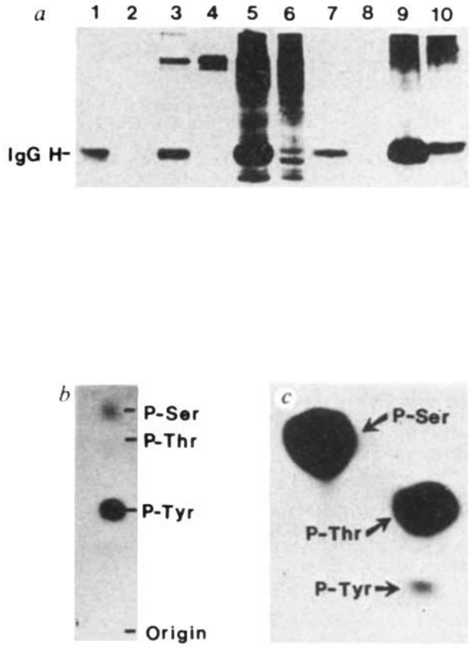

Fig. 3 *Drosophila* possess tyrosine-specific protein kinase activity. Immune complex kinase assays were performed as described previously[3]. Extracts were made from Rat 2 and K_c cells as described elsewhere[3]. Extracts from *Drosophila* embryos, larvae and adults were prepared in the same manner except that the samples were disrupted in a Dounce homogenizer. Approximately equal amounts of crude extract protein were assayed in the reactions represented by individual lanes in *a*. The position of the immunoglobulin heavy chain (IgG-H) was visualized by staining with Coomassie blue. The lanes are: 1, Rat 2 cells with tumour serum 2; 2, Rat 2 cells with normal serum; 3, K_c cells with tumour serum 2; 4, K_c cells with normal serum; 5, embryos with tumour serum 2; 6, embryos with normal serum; 7, larvae with tumour serum 2; 8, larvae with normal serum; 9, adults with tumour serum 2; 10, adults with normal serum. The IgG-H band from lane 3 was analysed for phosphoamino acid content as described by Hunter and Sefton[4] except that following hydrolysis phosphoamino acids were purified by ion exchange[24] and electrophoresed on cellulose thin-layer plates at pH 3.5 for 90 min at 600 V. The plates were dried and autoradiographed for 1 day before the detection of markers by ninhydrin staining. The result is shown in *b*. *c* Shows a two-dimensional phosphoamino acid analysis of K_c cells. Approximately 10^7 cells were labelled for 3 h with 5 mCi of radioactive orthophosphate in 300 μl of D-20[25] media lacking phosphate. The cells were lysed in 0.3% SDS, 1% 2-mercaptoethanol, 50 mM Tris (pH 7.4) 5 mM $MgCl_2$, 100 μg ml^{-1} DNase I and 50 μg ml^{-1} RNase A. After 10 min at 0 °C, the proteins were precipitated by the addition of trichloroacetic acid to 15%. The proteins were hydrolysed and analysed as described by Cooper and Hunter[24]. Markers were detected by ninhydrin staining. The radioactive spots were scraped into scintillation vials and counted in a toluene-based fluor.

prediction using antisera raised in newborn rabbits against RSV-induced tumours[3,19]. All the tumour antisera immunoprecipitate pp60^{v-src}. The antisera do, however, have varying affinities for pp60^{c-src} (ref. 9). When an immune complex containing either pp60^{v-src} or pp60^{c-src} is incubated with ATP, a tyrosine of the immunoglobulin heavy chain is phosphorylated by the *src* protein[2–6,9,10]. The results of such an immune complex kinase assay performed with extracts from Rat 2 cells, *Drosophila* K_c cells, and *Drosophila* embryos, larvae and adults are shown in Fig. 3*a*. Tumour serum 2 recovered detectable kinase activity from all of the extracts, whereas normal adult rabbit serum did not. Five of seven tumour sera tested detected kinase activity in K_c cells (data not shown). The two tumour sera which failed to detect kinase activity are specific to pp60^{v-src} and do not immunoprecipitate pp60^{c-src}. Phosphoamino acid analysis of the IgG chains phosphorylated by the K_c extract demonstrated that the phosphorylation was on a tyrosine residue (Fig. 3*b*).

The immune complex kinase assay is not a direct test of activity *in vivo*. We have, therefore, examined K_c cells for the presence of phosphotyrosine in order to demonstrate that tyrosine-specific protein kinases actually function in *Drosophila* cells. Figure 3*c* shows a two-dimensional phosphoamino acid analysis of ^{32}P-labelled K_c cells. Phosphotyrosine represented 0.1% of total phosphoamino acids. A value of 0.01% has been determined for Rat 2 cells using the same methods and is typical of untransformed cells (ref. 4 and our unpublished results). *Drosophila* cells must therefore contain tyrosine-specific protein kinases.

We have not yet been able to attribute the tyrosine-specific protein kinase activity in our immunoprecipitates directly to either a particular polypeptide or any of the three loci that contain homology to v-*src*. However, the tumour antisera used in this study do not react with tyrosine-specific protein kinases other than pp60^{v-src} and pp60^{c-src}. It is therefore likely that the tyrosine-specific protein kinase in *Drosophila* cells that is immunoprecipitated by these antisera is encoded by at least one of the three loci that we have cloned. We are now examining adult flies that are aneuploid for these chromosomal regions for gene dosage dependence of immune complex kinase activity.

Much of what is known about the role of pp60^{v-src} in neoplastic transformation has been gained either by studying cells before and after RSV-induced transformation or by studying conditional mutants of v-*src*. The role of pp60^{c-src} in normal cellular physiology has remained an enigma largely due to the absence of similar genetic approaches. The presence of c-*src* and tyrosine-specific protein kinases in *Drosophila* may facilitate genetic analysis of the role of c-*src* in normal cells.

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Dispersion of the *ras* family of transforming genes to four different chromosomes in man

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Cellular transforming genes (c-*onc*) are evolutionarily conserved vertebrate DNA segments which have been identified by two different approaches. One group of these cellular genes has been defined by their close homology to the transforming genes of the acute transforming retroviruses (v-*onc*)[1–3]. The second group, which represent activated forms of normal cellular genes[1,4–9], has been detected by the ability of certain genes from animal and human tumours to induce focal transformation of tissue culture cells. Investigation of the possibility that the same cellular gene might have given rise to both a retroviral and a tumour transforming gene revealed that two of the c-*onc* genes identified by transfecting genomic DNA from human tumours to murine 3T3 fibroblasts were related to the transforming genes of two closely related acute transforming retroviruses, Harvey murine sarcoma virus (HaMuSV) and Kirsten murine sarcoma virus (KiMuSV)[10–12]. The transforming genes of HaMuSV and KiMuSV are derived from two members of a cellular *onc* gene family called *ras*, which is a rather divergent group of normal vertebrate genes originally found by analysis of the cellular homologues of the v-*onc* genes of HaMuSV and KiMuSV[13]. Four distinct human cellular homologues of v-Ha-*ras* and v-Ki-*ras* (designated c-Ha-*ras* and c-Ki-*ras*, respectively) have been characterized[14]; two (c-Ha-*ras*-1 and c-Ha-*ras*-2) are more closely related to v-Ha-*ras*, while the others (c-Ki-*ras*-1 and c-Ki-*ras*-2) are more closely related to v-Ki-*ras*. On ligation with a retroviral long terminal repeat, the c-Ha-*ras*-1 gene of both rat and human have been shown to induce *in vitro* transformation of mouse NIH 3T3 cells by DNA transfection[15,16]. This gene and c-Ki-*ras*-2 have also been isolated as activated transforming genes in human tumours[10–12]. An understanding of the genetic relationship of the c-*ras* genes and additional genetic loci possibly involved in neoplastic transformation would be greatly facilitated by placement of the *ras* genes on the human chromosome map. Using DNA analysis of rodent × human somatic cell hybrids, we have now assigned each of the human genes to a different chromosome.

Somatic cell hybrids were constructed between fresh human lymphocytes (LLL) and rodent cells (mouse RAG and Chinese hamster E36) which were mutant in their hypoxanthine phosphoribosyl transferase gene, permitting hybrid selection on hypoxanthine–aminopterin–thymidine medium. Nine independent fusions were performed using PEG 1000, and 249 hybrids were derived (78 RAG × LLL and 161 E36 × LLL). These hybrids retained the entire rodent genome but segregated human chromosomes in different combinations. Following isozyme analysis of each of these primary hybrids[17], two mapping panels (16 hybrids with RAG and 30 hybrids with E36) were chosen, selecting hybrids with low numbers of human chromosomes, but with strong isozyme signals for the human enzymes. For a given hybrid, high molecular weight DNA, isozyme extracts and karotypic spreads were prepared at the same cell passage. G-11 chromosome staining was performed on each hybrid in the panel to discover hybrids having numerous chromosome rearrangements. This procedure stains human chromosomes light blue and rodent chromosomes magenta[18]. Hybrids with numerous human chromosome rearrangements or interspecific chromosome translocations were discarded. Each hybrid in the panel was analysed for up to 36 isozyme markers previously mapped to human chromosomes[17]. In addition, each hybrid was G-banded and the human chromosome complement determined[19].

The c-Ha-*ras*-1 gene was visualized as a 2.9-kilobase (kb)-fragment in human DNA following digestion with *Sac*I (Fig. 1*a*), electrophoresis in 0.6% agarose gels, transfer to nitrocellulose and hybridization to a nick-translated probe derived from a molecularly cloned 2.9-kb human *Sac*I fragment which includes the human c-Ha-*ras*-1 locus[14]. A *Sac*I digestion of E36 DNA did not produce any hybridization in this region of the filter. A lower stringency hybridization to a *Sac*I digest using the same probe revealed an additional 12-kb fragment in human DNA (c-Ha-*ras*-2 see below) and the Chinese hamster c-Ha-*ras* homologue (2.3 and 0.5 kb) in E36 DNA. Thus, by *Sac*I digestion of DNAs from the hybrid panel, it was possible to determine which hybrids contained the human c-Ha-*ras*-1 locus. The presence of c-Ha-*ras*-1 showed perfect concordance with the presence of human chromosome 11, with *LDHA* (lactate dehydrogenase) and with *ACP2* (acid phosphatase-2), isozyme markers previously mapped to chromosome 11 (Table1). Each of the remaining 22 human chromosomes showed high discordance with c-Ha-*ras*-1 (≥38%). These data, which indicate that c-Ha-*ras*-1 is localized on human chromosome 11, independently confirm this assignment already made by others[20,41].

A 3.6-kb *Bam*HI fragment characteristic of the c-Ha-*ras*-2 was detected in Southern transfer of human DNA by hybridization to a nick-translated probe derived from a cloned 0.7-kb *Bal*I fragment of human c-Ha-*ras*-2 (Fig. 1*b*). This fragment contained nearly the entire c-Ha-*ras*-2-specific segment and approximately 100 additional bases of human flanking DNA which are not homologous to v-Ha-*ras* (see Fig. 1*b*). A low stringency hybridization of a *Bam*HI digest of human DNA detects c-Ha-*ras*-1 (6.6 kb, see Fig. 2*a*, map) but does not detect the Ki-*ras* loci[14]. A *Bam*HI digest of E36 DNA revealed three bands (3.9, 4.5 and 8.6 kb) which did not co-migrate with the human signal, permitting detection of human c-Ha-*ras*-2 in the hybrids. The c-Ha-*ras*-2 gene was 100% concordant with the X chromosome and discordant (≥40%) with the other 22 chromosomes (Table 1). These results permit the assignment of c-Ha-*ras*-2 to the human X chromosome. One of the hybrids, 81P1, was positive for both *G6PD* (glucose 6-phosphate dehydrogenase) and *HPRT*, but negative for c-Ha-*ras*-2 and for the X chromosome by banding. As these two markers are both located near the terminus of the long arm of the human X chromosome (*G6PD*-q28; *HPRT*-q26-q28, it seems that a centromere-proximal (to q26) region of the X contains c-Ha-*ras*-2. This region was lost in hybrid 81P1 and the X terminus (q26-q28) has been translocated to another chromosome, which we could not detect in our chromosome analyses.