cells; however, the purifications have been linearly scaled up so that multiple frozen pellets can be extracted, pooled, and worked up. The individual recombinant proteins appear to have the same biochemical properties as the isotypes purified from bovine brain and as such these recombinant proteins can be employed for various PKC studies.

[56] Construction and Expression of Linker Insertion and Site-Directed Mutants of *v-fps* Protein-Tyrosine Kinase

By JAMES C. STONE, MICHAEL F. MORAN, and TONY PAWSON

Introduction

Several strategies are available for creating mutations in molecularly cloned oncogenes and protein kinase cDNAs,¹ each with its attendant strengths and weaknesses. The approach must depend on the specific questions being asked: What protein determinants are required for biological activity and which residues are dispensable? What are the limits of individual functional domains? What is the significance of a particular residue or sequence motif that has been conserved in evolution? How do the domains interact? Perhaps most important, with what other cellular proteins (such as substrates and regulators) does the protein kinase interact to induce a cellular response?

To identify the functional domains of the P130^{*gag-fps*} protein-tyrosine kinase, encoded by the *v-fps* oncogene of Fujinami avian sarcoma virus, we exploited a simple and generally applicable method to generate numerous defined lesions throughout the *v-fps* coding sequence.² Mutants were then expressed in mammalian or bacterial cells. The elements of this approach are defined below.

In-Phase Linker-Insertion Mutagenesis: General Strategy for Genetic Dissection of Complex Polypeptides

General Strategy

The basic approach is to generate linear plasmids by partial digestion with restriction enzymes that recognize numerous positions throughout

¹ M. Smith, *Annu. Rev. Genet.* **19**, 423 (1985).

² J. C. Stone, T. Atkinson, M. Smith, and T. Pawson, *Cell (Cambridge, Mass.)* **37**, 549 (1984).

the region of interest, to attach small duplex DNA molecules designed to leave the reading frame of the target coding sequence unaltered, to circularize these variant plasmids *in vitro*, and to recover and map these insertion mutations in *Escherichia coli* prior to functional analysis in vertebrate cells. The net effect of these manipulations is to create many different mutant proteins, each with a small peptide insertion at a distinct site in the polypeptide chain. Such insertions can have profound effects on protein function, and can give clues as to the limits, activities, and interactions of polypeptide domains. This technique serves as a basis for subsequent, more detailed analysis by oligonucleotide-directed deletion, point and cassette mutagenesis. Although originally developed to investigate an oncogenic tyrosine kinase, this approach has been successfully applied by us to the *c-raf* serine/threonine protein kinase³ and to the *c-myc* nuclear protein,⁴ and by others to a variety of proteins, including protein kinases⁵⁻⁹ and proteins involved in cellular or viral transcription and replication.¹⁰⁻¹³

Construction of the Vector for Mutagenesis

Substantial effort should be invested in design of the vector. The relevant cDNA should be inserted into a high-copy plasmid such as pUC.¹⁴ Construction of mutations in a plasmid that serves as a eukaryotic expression vector facilitates subsequent gene transfer and analysis without recourse to subcloning. Incorporation of a simian virus 40 (SV40) replication origin into the plasmid allows mutant proteins to be transiently expressed to high levels in COS monkey cells (see below). This type of analysis is extremely valuable, since one can rapidly verify that a full-length protein is made, and assess its stability and kinase activity. Useful vectors for this

³ U. R. Rapp, G. Heidecker, M. Huleihel, J. L. Cleveland, W. C. Choi, T. Pawson, J. N. Ihle, and W. B. Anderson, *Cold Spring Harbor Symp. Quant. Biol.* **53**, 173 (1988).

⁴ J. C. Stone, T. deLange, G. Ramsey, E. Jakobovits, J. M. Bishop, H. E. Varmus, and W. Lee, *Mol. Cell. Biol.* **7**, 1697 (1987).

⁵ M. Ng and M. L. Privalsky, *J. Virol.* **58**, 542 (1986).

⁶ R. Prywes, J. G. Foulkes, and D. Baltimore, *J. Virol.* **54**, 114 (1985).

⁷ E. T. Kipreos, G. J. Lee, and J. Y. J. Wang, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1345 (1987).

⁸ J. E. DeClue and G. S. Martin, *J. Virol.* **63**, 542 (1989).

⁹ S. D. Lyman and L. R. Rohrschneider, *Mol. Cell. Biol.* **7**, 3287 (1987).

¹⁰ V. Giguere, S. M. Hollenberg, M. G. Rosenfeld, and R. M. Evans, *Cell (Cambridge, Mass.)* **46**, 645 (1986).

¹¹ M. L. Privalsky, P. Boucher, A. Koning, and C. Judelson, *Mol. Cell. Biol.* **8**, 4510 (1988).

¹² L. Zumstein and J. C. Wang, *J. Mol. Biol.* **191**, 333 (1986).

¹³ V. R. Prasad and S. P. Goff, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3104 (1989).

¹⁴ C. Yanisch-Perron, J. Vieira, and J. Messing, *Gene* **33**, 103 (1985).

purpose include pECE¹⁵ and pCMV.¹⁶ The vector may also include a selectable marker, such as *neo*^r,¹⁷ to allow for selection of stably transfected mammalian cells (i.e., pIV2.3 in Fig. 2). However, the efficiency with which mutations are recovered in the target coding sequence depends on the relative proportion of this sequence in the plasmid, which will obviously be decreased by inclusion of a selectable marker. If possible, we recommend the use of cotransfection with a selectable marker plasmid such as pSV2*neo*¹⁷ for the isolation of stably transfected cells. If one anticipates using techniques such as electroporation or microinjection, a plasmid vector including an internal selectable marker is more suitable, especially in the analysis of biologically defective mutants. For retroviral transfer, it may be simplest to do the mutagenesis using a cassette containing the cDNA of interest, and to insert the individual mutants into a retroviral vector, as described for linker insertion mutagenesis of *v-src* by DeClue and Martin.⁸ Since the actual construction and mapping of mutations in the plasmid is straightforward, emphasis should be placed on constructing the vector that best suits the functional tests anticipated. Since tyrosine kinases and many other eukaryotic enzymes are active following expression in *E. coli*, it can be advantageous to do the initial mutagenesis and screening in a bacterial system, and then to transfer interesting mutants into a mammalian expression vector (see Refs. 7 and 13, and Fig. 2). Using pUC-based plasmids we rarely obtain mutations within the bacterial plasmid backbone, presumably because these tend to abolish antibiotic resistance or plasmid replication.

Design of the Mutagenic Oligonucleotide

Mutagenic oligonucleotides are synthesized based on the following criteria. The linker should insert a multiple of 3 bases so as not to disturb the reading frame, and must contain a restriction site for an enzyme that does not cut the parental plasmid. Some enzymes have unfavorable properties, such as inhibition by contaminants commonly found in crude plasmid preparations, and should be avoided. In principle a complementary hexanucleotide can be employed,¹⁸ although we have been unsuccessful with several of these. A dimer of a 6-bp recognition sequence, on the other hand, can be attached to the blunt ends of linear plasmids with high efficiency. For example, the 12-mer 5'-CTCGAGCTCGAG-3' has two

¹⁵ L. Ellis, E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter, *Cell* (Cambridge, Mass.) **45**, 721 (1986).

¹⁶ D. Russell, personal communication.

¹⁷ P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982).

¹⁸ F. Barany, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4202 (1985).

FIG. 1. Linker-insertion mutagenesis. A plasmid is linearized with a blunt-end cutter (*Rsa*I), and a nonphosphorylated dodecameric linker with novel *Xho*I and *Sst*I sites is introduced, creating an insertion of four codons. The insertion can be reduced to a hexamer (monolinker) encoding only two extra codons, with the loss of the *Sst*I site, by digestion with *Xho*I. Variations on this theme are discussed in the text.

*Xho*I recognition sites (5'-CTCGAG-3'), and an internal *Sst*I site (5'-GAGCTC-3') where the two *Xho*I sites meet (Fig. 1). Depending on the exact protocol followed, one can selectively recover mutations with an insertion of one, two, or multiple copies of the hexameric (5'-CTCGAG-3') *Xho*I sequence, creating what we refer to as mono-, di-, and polylinker mutations, respectively. The absence of the *Sst*I site can generally be used to distinguish monolinker mutations from other alleles.

A second type of mutagenic linker we have successfully employed is typified by the partially self-complementary sequence 5'-CGACCTCGAGGT-3'. This dodecamer is designed to attach to pro-

truding 5'-CG ends, and to specify an *Xho*I site (underlined). In this case a single 12-bp insertion would constitute a monolinker mutation, and a novel *Sal*I site is diagnostic of multiple insertions. While hexamers can be efficiently attached to recessed ends,¹⁸ the use of a 12-base mutagenic oligonucleotide allow one to insert the same recognition sequence (i.e., *Xho*I) into all sites.

The linker should be designed to avoid stop codons in any reading frame. While large linker insertions might generally be expected to give a more severe phenotype, there may be cases where this is not so; a tetrapeptide insertion in an α -helical region might give less angular distortion than a dipeptide, for example. Clearly one is not limited to the linkers described above. The sequence of the linker can be tailored to suit any enzyme used to linearize the DNA, or to introduce any novel restriction site (with the caveat that some such linkers may encode translational termination signals in one or more reading frames). Linkers encoding specific structural motifs, rather than random oligopeptides, can also be designed.¹⁹ Obviously the quality of the chemistry employed in the synthesis of the mutagenic oligonucleotide is crucial if one is to avoid mutations of unexpected structure.

Partial Digestion of the Plasmid

Starting with a plasmid preparation which contains predominantly covalently closed circles and virtually no linear molecules, conditions are established to optimize the yield of linear plasmid by partial digestion. *Alu*I, *Dpn*I, *Fnu*DII, *Hae*III, and *Rsa*I are enzymes with tetranucleotide recognition sequences which generate blunt ends suitable for attaching the *Xho*I dimer. *Hin*PI, *Taq*I, and *Msp*I all generate 5'-CG overhangs, which can be attached to the partially self-complementary dodecamer described above. When initial functional tests indicate that a particular region of the protein is interesting, it is often possible to increase the local density of mutations by locating sites for single-cutting enzymes. In theory, and usually in practice, it is possible to create an in-phase insertion at any restriction site by modification of the ends of the linear plasmid or by synthesizing appropriate oligonucleotides.

Partial digestion giving adequate yields of linear plasmid DNA can be achieved either by performing the digestion in the presence of ethidium bromide, or by limiting both the duration of the incubation and the concentration of the restriction enzyme. There seems to be no advantage to the ethidium method, and the latter technique is simpler. In either case the

¹⁹ D. Periman and H. O. Halvorson, *Nucleic Acids Res.* **14**, 2139 (1986).

conditions are empirically determined as described in the protocol below. To monitor plasmid digestion it is imperative to use electrophoresis conditions that adequately resolve open circles, covalently closed circles, full-length linear species, and linear species of less than full length that arise from multiple cleavages. It is not necessary to isolate linear species from the partially digested plasmid prior to linker attachment. Ligations are performed on the mixed products of a partial enzyme digestions, and the linear plasmid with attached linkers is subsequently isolated and introduced into *E. coli*.

Protocol for Linker-Insertion Mutagenesis

For detailed descriptions of recombinant DNA procedures the reader is referred to Sambrook *et al.*²⁰

1. Restriction enzyme (2–4 units) is added to a tube containing 50–100 μg of plasmid in 100 μl of the appropriate buffer. If the enzyme is very concentrated it may be advantageous to dilute 1–2 μl of the enzyme into ice-cold reaction buffer immediately before use. In this way the use of very small volumes can be avoided and the method can be readily reproduced.
2. Incubate the reaction at the recommended temperature for enzyme digestion.
3. At 2, 4, 8, 16, 32, and 64 min, remove 15 μl of the reaction to tubes containing 85 μl of TNE (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) and 100 μl of Tris-buffered phenol–chloroform–isoamyl alcohol (25 : 24 : 1) at room temperature. Vortex each sample briefly after the DNA sample is added.
4. After the last sample is removed from the digestion tube vortex all the samples vigorously, centrifuge at room temperature, and remove the aqueous phase to fresh tubes.
5. Precipitate each sample with ethanol. Add 0.1 vol of 3 M sodium acetate and 2 vol of 95% ethanol, vortex, and leave at -20° for 1 hr. Centrifuge each sample at maximum speed in a microfuge and decant the supernatants. Rinse the tubes with 70% ethanol and then with 95% ethanol. Spin the tubes briefly after each rinse if the pellets are not visibly secure on the bottoms of the tubes. Dry the pellets and dissolve each in 20 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).
6. Mix 1 μl of each sample with 9 μl of TE and 2 μl of sample buffer, and electrophorese in a 20-cm 0.5% agarose gel in Tris–acetate–EDTA buffer for 12–18 hr at 20 V. Run uncut plasmid and plasmid cut with an

²⁰ J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1989.

enzyme that cleaves only once as controls. It may be necessary to allow the gel to solidify for 1 hr at 4° prior to loading. Ethidium bromide can be included in the gel at 1.0 $\mu\text{g}/\text{ml}$ or the gels can be stained after electrophoresis.

7. Fractions of partially digested DNA that are rich in linear species and which show no signs of multiply cut plasmid are identified and can be pooled.

8. Linker is prepared as described by Lathe *et al.*²¹ Briefly, the non-phosphorylated oligonucleotide is dissolved in TE at a concentration of 0.5–1.0 $\mu\text{g}/\text{ml}$, heated to 65°, and allowed to cool slowly to room temperature to form duplex DNA. These preparations may be stored at –20° until use.

9. An aliquot of partially digested DNA corresponding to 1.0 μg of linear species is mixed with 1.0 μg of linker and adjusted to 9.0 μl with TE. DNA species are then coprecipitated by addition of 0.1 vol of 3 M sodium acetate and 3 vol of 95% ethanol.

10. The coprecipitated DNA is dissolved in 5.0 μl of TE by pipetting up and down and then allowing the tube to sit on ice for several minutes. Solution (4.5 μl) is transferred to a fresh tube and 0.5 μl of 10 $\times$ T4 DNA ligase buffer (200 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 50 mM dithiothreitol, 500 $\mu\text{g}/\text{ml}$ bovine serum albumin, 5 mM ATP) is added. T4 ligase is added (0.5 μl ; 2–3 Weiss units), and the reaction is incubated at room temperature for 4 hr. Blunt-end ligation proceeds much more efficiently at room temperature than at 4°. This reaction requires a high concentration of ligase, but the glycerol from the ligase storage buffer should not exceed 5% of the final volume. Since the linkers have 5' hydroxyl groups they cannot be ligated to one another, and can only be covalently attached to the phosphorylated 5' end of the linearized plasmid DNA.

11. After addition of sample buffer, the ligation reaction is heated to 65° for a few minutes, and then loaded on a 0.5% agarose gel as described above to separate linear molecules which should now have linkers attached.

12. The linear species is excised from the gel, and recovered by electroelution followed by ethanol precipitation.

13. After estimating the recovery of linear species by running a small sample on an agarose gel and staining with ethidium bromide, an aliquot corresponding to about 0.1 to 0.2 μg of linear DNA is diluted into 20 μl of TE. The sample is heated to 65° and allowed to cool slowly to promote recircularization. In this step, the covalently attached strands of the linker

²¹ R. Lathe, M. P. Kieny, S. Skory, and J. P. Lecocq, *DNA* 3, 173 (1984).

will hybridize, although the plasmid is not yet covalently closed. The plasmid DNA molecules are then transformed into *E. coli* using standard procedures, where the circles are closed and the plasmids replicate. The choice of *E. coli* strain does not seem critical. This procedure will result in the insertion of a single linker. In the case of the *XhoI* dimer dodecameric linker attached to blunt-ended DNA, the size of the linker can be reduced to a hexameric ("monolinker") 5'-CTCGAG-3' insertion by subsequent digestion of mutant plasmid DNA with *XhoI*, followed by religation and transformation of *E. coli* (Fig. 1).

Variations

1. If polylinker mutations are desired, the linker must be phosphorylated before the coprecipitation step. Immediately after ligation, digestion with the linker-cleaving enzyme (i.e., *XhoI*) is required to convert plasmid-linker concatamers to species of plasmid unit length, to reduce the number of attached linkers, and to generate cohesive ends. If the digestion is thorough, monolinker mutations are generated by this method. In practice the digestion of linker is incomplete and polylinker insertions are recovered. After isolating the linear species from the gel, an aliquot of this DNA is converted to covalently closed circular form using T4 DNA ligase, followed by transformation into *E. coli*.

2. Ethidium bromide can be included in the initial enzymatic digestion to inhibit multiple cutting, and thereby promote the formation of linear molecules. Plasmid DNA (2 μ g), 1 unit restriction enzyme, and 0.1 to 20 μ g ethidium bromide are incubated in 20 μ l containing 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM MgCl₂ at 37° for 60 min. The concentration of ethidium bromide and enzyme that gives predominantly linear molecules is empirically determined as above. This procedure has been extensively described.^{2,5}

Identification and Mapping of Linker Mutations

Linker-containing plasmids are identified and roughly mapped within the target sequence by a pair of double restriction enzyme digests, each using single cutters that cleave outside the region of interest, in addition to the enzyme that cuts within the linker. Mutants will contain a novel restriction site specified by the linker, whose approximate position is given by the sizes of the digestion products. Although rapid and simple, the following problems must be considered in mapping the mutations.

1. A cautionary point is that the original preparation of linear species used to transform *E. coli* is invariably contaminated with plasmid cleavage

products of slightly less than full length, arising from double digestion at two neighboring sites in the plasmid. The consequence of this double cleavage, if followed by linker attachment, is a small deletion that may not be apparent from the initial mapping experiments described above. Digestion of all putative linker insertion mutations with an enzyme that does not cut the linker, but rather cuts the parental plasmid into several small fragments in the 3.0- to 0.5-kb range, allows identification of such unwanted deletion plasmids.

2. Small amounts of covalently closed circles contaminating the original linear preparation will seriously decrease the percentage yield of mutant plasmids, since uncut plasmid is recovered in *E. coli* with high efficiency relative to mutated plasmid.

Both these difficulties can be reduced or eliminated by carefully controlling the partial digestion and preparative electrophoresis conditions. High-resolution mapping using molecules end labeled on the *Xho*I-generated termini allows one to deduce the exact positions of the mutations. Alternatively, the mutant structures can be determined directly by DNA sequence analysis, using a set of oligonucleotide primers for dideoxynucleotide sequencing spaced at 200-bp intervals throughout the region of interest. We find that the sequence of the mutants invariably conforms to that expected from the initial mapping. Ultimately the quality of the synthetic oligonucleotide and the enzymes employed determine the predictability of the insertion mutations.

A plasmid that contains di- or polylinker insertions can be converted to the monolinker form by exhaustive digestion with the linker-cleaving enzyme. The linear plasmid is then circularized using ligase, and introduced back into *E. coli*. It is simple to screen for the reduction in linker size by DNA sequencing, although the loss of the internal restriction site (*Sst*I or *Sal*I in the examples above) is generally diagnostic of a monolinker. A rare complication in this latter test arises when the restriction site diagnostic of di- or polylinker mutations is created at the boundary of the insertion. For example, when the *Xho*I dimer described above is attached to an *Alu*I site, an *Sst*I site should be created 6/16 times on a random basis, and hence the monolinker insertion mutant will still retain an *Sst*I recognition sequence at the mutation site.

Generating Deletion Mutants

An advantage of linker insertion mutants is that they can be used to generate additional mutants. Deletions can readily be constructed by digesting two different linker insertion mutants with the enzyme specified by the linker, and a single cutter elsewhere in the plasmid. If the two linker

insertions are in frame, appropriate DNA fragments from the mutants can simply be recombined. If they are in different reading frames, one can generally modify the ends of the plasmid cleaved at the linker to generate an in-phase deletion. For example, the 3' recessed ends of *Xho*I-generated linear DNA are filled out using Klenow polymerase; ligation of two such ends results in a +1 shift in reading frame. Digestion with single-strand-specific mung bean nuclease generates a -1 shift. Thus any pair of *Xho*I sites can be recombined in frame. Alternatively one can cut at the novel restriction site provided by the linker and digest with the slow isozyme of *Bal*31 exonuclease to generate a series of deletion variants.²² These deletion methods were used with particular advantage in the definition of functional protein domains crucial to *c-myc* transforming activity.⁴

Alternative Strategies for Insertion Mutagenesis

Several other attractive insertion mutagenesis methods have been developed. One approach employs a DNA fragment containing a marker which can be scored in *E. coli*, such as *supF*,²³ rather than a linker. Subsequently the marker is removed, leaving a small insert of foreign DNA. This method has some drawbacks, including (1) the preparation of the mutagen is much more laborious, (2) the procedure requires two rounds of plasmid manipulation, and (3) the minimal insertion is generally larger than 6 bp. The advantage of this technique is that virtually 100% of the plasmids analyzed contain inserts. However, since the protocol detailed above often yields greater than 80% mutant plasmids, the need for a selectable marker is obviated.

For genes that are small relative to the size of the plasmid it can be difficult to obtain a collection of linker insertions by the methods described here. The more cumbersome linker-scanning method, whereby a set of 5' and a set of 3' deletions are recombined using a common linker, achieves nearly the same net result.²² In this case the reading frame is not dictated by the enzymology, however, but rather is controlled by recombining appropriate pairs of plasmids after DNA sequence analysis.

Transient Expression of Wild-Type and Mutant *v-fps* Proteins in COS Monkey Cells

Transient Expression in COS Monkey Cells

If the linker insertion mutagenesis has been done in a plasmid vector containing an SV40 origin of replication, the activities of mutant proteins

²² L. I. Lobel and S. P. Goff, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4149 (1984).

²³ B. M. Willumsen, A. Christensen, N. L. Hubbert, A. G. Papageorge, and D. R. Lowy, *Nature (London)* **310**, 583 (1984).

can be rapidly assessed in COS monkey cells. COS cells constitutively synthesize the SV40 large T antigen, which provokes the extrachromosomal replication of circular DNA molecules with an SV40 replication origin, and hence the high level synthesis of any protein encoded by the plasmid. Transfection can be carried out using a variety of procedures, including calcium phosphate coprecipitation,^{24,25} DEAE-dextran-mediated transfection,²⁶ or lipid-mediated transfection²⁷ (lipofection). Two of these techniques are described below. See Sambrook *et al.*²⁰ and Freshney²⁸ for further details and discussion on transfection and tissue culture, respectively.

Protocol for Calcium Phosphate Transfection of COS Cells

COS-1 cells²⁹ are maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). They are seeded at approximately $5-10 \times 10^5$ cells/100-mm dish (approximately one-tenth of a confluent plate) and then transfected the next day essentially according to Graham and Van der Eb.²⁴ Use 10 μ g of plasmid DNA that has been purified by equilibrium centrifugation in a CsCl-ethidium bromide density gradient. It is important to optimize the pH and DNA concentration which give the best yield.

Reagents

2 $\times$ HEPES-buffered saline (2 $\times$ HBS): 1.64 g NaCl (280 mM), 1.19 g HEPES (58 mM), 21.3 mg Na₂HPO₄ (1.5 mM); dissolve in distilled H₂O to a volume of 100 ml and adjust the pH to 7.10 with NaOH.

Sterilize through a 0.22- μ m filter

CaCl₂ (2.5 M): Dissolve 18.4 g CaCl₂ · 2H₂O in distilled H₂O to a volume of 50 ml and sterilize through a 0.22- μ m filter

1. In a 5-ml sterile polystyrene tube, combine

Tris-HCl (1 mM) (0.5 ml), 0.1 mM EDTA, pH 8.0 containing 10 μ g plasmid DNA

CaCl₂ (2.5 M) (50 μ l)

In a 15-ml sterile conical polystyrene tube place 0.5 ml 2 $\times$ HBS.

2. Bubble sterile air into the 2 $\times$ HBS through a plastic 1-ml pipette

²⁴ F. L. Graham and A. J. Van der Eb, *Virology* **52**, 456 (1973).

²⁵ C. Chen and H. Okayama, *Mol. Cell. Biol.* **7**, 2745 (1987).

²⁶ J. H. McCutchan and J. S. Pagano, *J. Natl. Cancer Inst. (U.S.)* **41**, 351 (1968).

²⁷ P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7413 (1987).

²⁸ R. I. Freshney, "Culture of Animal Cells." Alan R. Liss, New York, 1983.

²⁹ Y. Gluzman, *Cell (Cambridge, Mass.)* **23**, 175 (1981).

attached to a Pipet-Aid (Drummond Scientific). Slowly, and dropwise, add the Ca^{2+} -DNA solution into the phosphate solution using a 1-ml blue-tipped pipettor. Let the precipitate accumulate at room temperature for 20 min (the precipitate may not be very obvious).

3. Pipette the mixture onto the COS-1 cells using a plastic pipette and gently swirl the plate to evenly distribute the precipitate.

4. The next day (16 hr later) rinse the cells once with warm phosphate-buffered saline (PBS) and then feed with fresh 10% FBS-DMEM.

5. Two days later the cells should be expressing the plasmid-encoded protein in substantial amounts. The transfected gene product can be detected by Western blotting or by immunoprecipitation, and analyzed for kinase activity following immunoprecipitation or partial purification.

We have not routinely employed dimethylsulfoxide (DMSO) or glycerol "shocking" or chloroquine treatment to enhance DNA uptake by COS cells or Rat-2 cells,²⁰ or the modified calcium phosphate technique of Chen and Okayama.²⁵ Such additional steps may ultimately increase the expression of the plasmid-encoded protein when using the calcium-phosphate method.

Lipofection of COS Cells

As an alternative to introducing plasmid DNA into COS cells as a calcium-phosphate precipitate, we have used Lipofectin reagent (BRL, Inc., Bethesda, MD). This is a 1:1 solution of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride and dioleoylphosphatidylethanolamine as described by Felgner *et al.*²⁷ In this method, the DNA is first mixed with the lipid, giving a lipid-DNA complex that can apparently fuse with the cells. We have found this method to be very simple and reproducible, although it is expensive. However, we have experienced batch-to-batch variations in the Lipofectin reagent which can affect cytotoxicity. New lots of the reagent should be optimized for the amount of Lipofectin and the duration of exposure which give maximal transfection efficiency and minimal cell death.

Method

1. COS-1 cells are seeded at approximately 2×10^5 /60-mm dish the day before the transfection.

2. Plasmid DNA (1.0 μg) is combined with 1.5 ml of serum-free, growth factor-supplemented medium (Opti MEM I; BRL, Inc.) containing 55 μM 2-mercaptoethanol in a sterile polystyrene tube (polypropylene tubes should be avoided). In a second polystyrene tube combine 25 μg of the

lipid reagent in 1.5 ml of the above medium. Combine the diluted DNA and lipid solutions and mix gently (do not vortex) and let stand at room temperature for 15 min.

3. Wash cells three times with warm PBS lacking Ca^{2+} and Mg^{2+} . It is essential that all traces of serum are removed from the cells. Add the 3 ml of DNA-lipid mixture to the cells and incubate for 5 hr.

4. After the 5-hr incubation add to the cells 3 ml of DMEM containing 20% FBS such that the final serum concentration is brought to 10%.

5. The next day (approximately 16 hr later) change to 5 ml of fresh DMEM containing 10% FBS.

6. Two days later the cells may be harvested for analysis of protein kinase expression and activity.

Stable Expression of Mutant Protein Kinases in Rat-2 Fibroblasts

To obtain stable cell lines expressing wild-type or mutant forms of P130^{*gag-fps*}, we have generally employed the Rat-2 fibroblast cell line.³⁰ This line has several useful characteristics. It lacks endogenous thymidine kinase activity, meaning that the *tk* gene can be used as a coselectable marker if required. Rat-2 cells can be transfected with good efficiencies (approximately 1 : 10³ stable transfectants), arrest their growth when confluent, and virtually never give rise to spontaneously transformed colonies. This is an important consideration when transforming activity is being measured. With the *gag-fps* oncogene, it is obviously possible (and desirable) to measure transforming efficiency in monolayers of transfected Rat-2 cells. Transformed foci can be readily picked, expanded, and cloned by growth in soft agar or by limiting dilution. Such cells invariably express the transfected oncogene at high levels. However, to study mutants with little or no transforming activity, or to express protein kinases or other signaling proteins with no dramatic effects on cell morphology or growth properties, it is necessary to select transfectants on the basis of a cotransfected marker and to screen for synthesis of the desired gene product. Resistance to the antibiotic G418 conferred by the *neo^r* gene is generally the selection procedure of choice.

Protocol for Isolation of Stably Transfected Cell Lines Using Coselection for G418 Resistance

This method allows for the screening of several G418-resistant colonies for expression of the transfected gene product with a minimum delay. Colonies are transferred into 12-well plates and analyzed by Western

³⁰ W. C. Topp, *Virology* **113**, 408 (1981).

blotting of whole-cell lysates. By screening for expression soon after the initial picking, positive and negative clones can be identified and negative isolates can be discarded before they have been needlessly expanded.

1. Approximately 2×10^6 Rat-2 cells are plated in a 100-mm dish in DMEM + 10% FBS and left overnight, or until about 30% confluent.

2. Cells are transfected by either of the two procedures described above. If the plasmid does not contain an internal selectable marker, a 10:1 ratio of the plasmid encoding the kinase to pSV2neo (which encodes the aminoglycoside phosphotransferase that confers resistance to the antibiotic G418) is employed. With the calcium-phosphate method, 1 μ g of plasmid (combined with 0.1 μ g of pSV2neo if necessary) and 9 μ g of sheared Rat-2 genomic DNA (as carrier) are used. The Rat-2 carrier DNA is not included when using lipid-mediated transfection. It is recommended that these DNAs be sterilized by ethanol precipitation before transfection when attempting to establish cell lines.

3. Two days after the transfection the cells are trypsinized and reseeded onto five plates in DMEM + 10% FBS and 400 μ g/ml G418 (1 $\times$ concentration) (Geneticin; GIBCO, Grand Island, NY).

4. After 2–3 days cells will begin to slough off of the monolayer. The G418-containing medium is changed every 2 days. Ten to 14 days after the transfection, colonies containing approximately 500–1000 G418-resistant cells should be visible.

5. At this point individual colonies can be isolated using cloning cylinders. The positions of up to 12 well-isolated colonies are marked on the underside of a plate with a marker pen. In a sterile tissue culture hood remove the medium and then rinse the cells gently with 5 ml of PBS, which is then aspirated from the plate. Using sterile forceps, place cloning cylinders onto the individual colonies according to the markings on the underside of the plate. Using a pipettor fitted with a sterile yellow tip, aliquot 50 μ l of 0.25% trypsin into each of the cylinders. Two to 4 min later, pipette the trypsin up and down within the cylinder to completely dislodge the cells from the surface of the plate. Care must be taken not to disturb the cylinder during this step. Finally, the cells are taken from the cylinder and placed into individual wells of a 12-well plate containing 1.5 ml of DMEM + 10% FBS + $1 \times$ G418.

6. Once the cells are confluent (approximately 4 days), they are rinsed with 1 ml of warm PBS, and then approximately 150 μ l (three drops) of 0.25% trypsin is added. Two to 4 min later 1 ml of DMEM + 10% FBS is added to the cells to inhibit the trypsin. A sterile blue-tipped pipettor is used to gently disperse the cells into the medium. One drop of the cell suspension is transferred into a fresh 12-well plate containing DMEM + 10% FBS + $1 \times$ G418 to propagate the clone. The rest of the cells are

placed into a snap-cap tube and pelleted by centrifugation for 2 min at 3000 rpm in a microfuge. (Note that this protocol is not suitable for transmembrane proteins that will be exposed directly to trypsin).

7. The supernatant is completely removed, and 30 μ l of SDS gel sample buffer is added to the cells. At this point the lysates may be stored at -20° for the extra 1–2 days that may be required for slower growing clones to accumulate.

8. Lysates are heated to 100° for 2 min, followed by vigorous vortexing to reduce their viscosity prior to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting. By using a mini-SDS–PAGE apparatus, and an antibody-alkaline phosphatase conjugate and color reaction to develop the Western blot, clones expressing the desired protein can be identified in 1 day. Positive clones can be identified in less than 3 weeks following the initial DNA transfection.

9. Positive clones are expanded from the second 12-well plate for further analysis and freezing. Cell lines should be maintained in medium containing at least 40 μ g/ml G418 to maintain the selective pressure on protein expression.

Analysis of Transforming Activity

1. To measure transforming activity cells are plated as above, and transfected using the calcium–phosphate procedure with 150 ng plasmid DNA and 10 μ g Rat-2 DNA/10-cm plate as described above.

2. Cells are allowed to grow to confluence, and the medium is switched to DMEM + 5% calf serum. The lower serum concentration favors the overgrowth of transformed cells with reduced growth factor requirements.

3. Cells are maintained for 2 weeks to 2 months. Cultures are monitored for the appearance of transformed foci, which can be quantitated as to foci formed per microgram of DNA transfected. For reasons that are unclear, poorly transforming alleles may induce foci on Rat-2 cells with a longer latent period than the wild-type gene, and hence it is worth keeping cells for a protracted period.

4. Foci of transformed cells can be picked either using a cloning cylinder (as above), or more readily with a sterile drawn-out Pasteur pipette. Picked cells are grown up, and can be cloned either by limiting dilution or by colony formation in soft agar (a property of many but not all morphologically transformed cells).

Specific Oligonucleotide-Directed Mutagenesis and Rapid Expression of *v-fps* Kinase Domain

Once a general view of the functional domains within a protein kinase has been obtained, it is possible to ask more specific questions about the

functions of individual residues or defined domains. For this purpose it is useful to create predetermined mutations using synthetic oligonucleotides. We have produced a variety of point, deletion, and insertion mutations in the viral and cellular *fps* and *fms* tyrosine kinases. These procedures have been described in detail elsewhere.³¹⁻³⁵ Two advances have made this procedure more rapid and efficient, especially if the mutant sequence must subsequently be expressed in mammalian cells. First, the coding sequence to be mutated can be placed into a plasmid containing an *f1* or M13 replication origin (phagemid), which can be propagated as a plasmid but rescued as single-stranded DNA at will following superinfection with a helper bacteriophage such as M13K07,³¹ providing a template for mutagenesis. The stability of inserts within such phagemids is generally greater than in M13 itself. Obviously it is helpful if the phagemid is designed to effect subsequent expression of the insert in mammalian cells (i.e., pCMV), since no subcloning of mutants is then necessary. Second, the template DNA can be prepared by growth of the bacteriophage or plasmid in *dut⁻ung⁻* cells (i.e., CJ236), which yield uracil-substituted DNA.³² On *in vitro* mutagenesis and introduction into wild-type *E. coli* the parental strand is selected against, and a very high frequency of mutants is produced. Usually mutants can be identified directly by DNA sequencing without recourse to prior screening procedures. For rapid analysis, the mutated sequences are subcloned into both bacterial and mammalian expression vectors (Fig. 2).

Discussion

Linker Insertion Mutagenesis Suggests a Domain Structure for the v-fps Tyrosine Kinase

In analyzing a collection of linker insertion mutations in the *gag-fps* coding sequence, some of which are shown in Fig. 3, we have recovered mutant proteins with the following phenotypes:

1. Indistinguishable from wild type

³¹ J. Viera and J. Messing, in "Methods in Enzymology" (R. Wu and L. Grossman, eds.), Vol. 153, p. 3. Academic Press, San Diego, California, 1987.

³² T. A. Kunkel, J. D. Roberts, and R. A. Zakour, in "Methods in Enzymology" (R. Wu and L. Grossman, eds.), Vol. 154, p. 367. Academic Press, San Diego, California, 1987.

³³ M. J. Zoller and M. Smith, in "Methods in Enzymology" (R. Wu and L. Grossman, eds.), Vol. 154, p. 329. Academic Press, San Diego, California, 1987.

³⁴ M. Moran, C. A. Koch, I. Sadowski, and T. Pawson, *Oncogene* 3, 665 (1988).

³⁵ G. R. Taylor, M. Reedijk, V. Rothwell, L. R. Rohrschneider, and T. Pawson, *EMBO J.* 8, 2029 (1989).

FIG. 2. Oligonucleotide-directed mutagenesis and expression of the *v-fps* tyrosine kinase. The *v-fps*-containing phagemid *pTZ1.4* are passed through the *dut*⁻, *ung*⁻ *E. coli* strain *CJ236*, and ssDNA is prepared by superinfection with *M13KO7*. Following *in vitro* mutagenesis using the uracil-substituted single-stranded template and a mutant oligonucleotide, the double-stranded heteroduplex is used to transform the *ung*⁺ strain *JM101* to ampicillin resistance. Mutants are identified by dideoxy sequencing. The mutations are then subcloned into bacterial or mammalian expression vectors. Mutant proteins are rapidly screened for kinase activity in bacteria and COS monkey cells, followed by analysis of transforming activity and recovery of stably expressing lines using Rat-2 fibroblasts.

LINKER INSERTIONS

POINT MUTANTS

FIG. 3. *gag-fps* linker insertion and point mutants generated using the techniques shown in Figs. 1 and 2. The kinase and transforming activities of the mutants, and the positions of the domains, are indicated (*, kinase activity not tested). Residues within the kinase domain which are highly conserved among protein kinases are in upper case, as is the major auto-phosphorylation site (Tyr-1073), which has been changed to a variety of different amino acids,^{34,38,39} none of which can functionally replace tyrosine. Two aspartic acid residues are conserved in all protein kinases and some aminoglycoside phosphotransferases,⁴⁰ and cannot be substituted with glutamic acid or asparagine without loss of activity.³⁴

2. Transformation defective, but positive for *in vitro* kinase activity
3. Temperature sensitive for both kinase and transforming activities
4. Host dependent for transforming activity
5. Transformation defective and kinase inactive

This broad range of mutant phenotypes, taken in conjunction with the locations of the insertion mutations and sequence comparisons of related gene family members, has allowed us to propose a domain structure for P130^{*gag-fps*} and has revealed previously unappreciated aspects of tyrosine kinase structure and function.

The most informative mutants are those that retain some aspect of their biological activity, or which lose activity unexpectedly. A series of adjacent mutations which induce similar phenotypes suggest a discrete functional domain made up of a contiguous stretch of the polypeptide backbone. For example, we described a series of insertions within the N-*fps* region of P130^{*gag-fps*} (Fig. 3) that dramatically decrease transforming activity in Rat-2 cells without any overt effect of kinase activity.² Subse-

quent analysis has suggested a role in subcellular localization.³⁶ We found an excellent correlation between mutations that perturbed the hypothetical N-*fps* functional domain and the failure of the encoded mutant proteins to react with two different anti-Fps monoclonal antibodies.³⁷ Apparently the antibodies recognize conformational epitopes within a domain whose structure is perturbed by any one of several insertions, resulting in a loss of biological, although not catalytic, activity.

The SH2 domain (Fig. 3)³⁸⁻⁴⁰ was also first identified and defined on the basis of linker insertions that abolished transforming activity.⁴¹ In contrast to the amino-terminal domain, however, these SH2 mutations inhibited kinase activity. This was a curious observation, since biochemical and structural evidence indicated that the catalytic domain is composed of residues entirely carboxy-terminal to SH2. We postulated that the SH2 region influences kinase activity through an intramolecular interaction with the catalytic domain. Consistent with this notion, SH2 insertions which affect kinase activity increase the sensitivity of the kinase domain to limited proteolysis, suggesting that they cause a substantial conformational change within the catalytic domain. Some SH2 mutations in avian *v-fps* and *v-src* induce a host range phenotype, transforming chicken but not rat cells.⁴² These and other observations have led to the speculation that the SH2 domain also influences substrate recognition, and may associate directly with other cellular proteins. The subsequent discoveries that phospholipase C- γ and p21^{ras} GTPase activating protein (GAP) contain SH2 domains, and are substrates for tyrosine phosphorylation, support the view that this protein substructure may play a key role in normal as well as oncogenic signal transduction.^{43,44}

Insertions in the *v-fps* or *v-src* catalytic domain most frequently result in a loss of kinase activity.^{2,8} Null mutations presumably identify critical regions or residues, although they tend to be otherwise rather uninformative. The identification of further mutations which restore activity to null alleles (pseudorevertants) may be useful in defining sites of intramolecular interactions. A host range *v-src* mutant with an insertion at the principal

³⁶ A. R. Brooks-Wilson, E. Ball, and T. Pawson, *Mol. Cell. Biol.* **9**, 2214 (1989).

³⁷ J. C. Stone and T. Pawson, *J. Virol.* **55**, 721 (1985).

³⁸ G. Weinmaster, M. J. Zoller, M. Smith, E. Hinze, and T. Pawson, *Cell (Cambridge, Mass.)* **37**, 559 (1984).

³⁹ G. Weinmaster and T. Pawson, *J. Biol. Chem.* **261**, 328 (1986).

⁴⁰ S. Brenner, *Nature (London)* **329**, 21 (1987).

⁴¹ I. Sadowski, J. C. Stone, and T. Pawson, *Mol. Cell. Biol.* **6**, 4396 (1986).

⁴² J. E. Declue, I. Sadowski, G. S. Martin, and T. Pawson, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 9064 (1987).

⁴³ T. Pawson, *Oncogene* **3**, 491 (1988).

⁴⁴ C. Ellis, M. Moran, F. McCormick, and T. Pawson, *Nature (London)* **343**, 377 (1990).

autophosphorylation site within the kinase domain has been identified.⁸ Clearly, insertions or other mutations in the kinase domain which induce a partial or complete loss of biological activities, but do not affect enzymatic activity per se (measured for example by autophosphorylation), may be useful in identifying important substrates. Ultimately it may be possible through the isolation of appropriate mutants to identify the kinase residues that actually contact substrates, allowing the definition of an "effector domain" as has been proposed from the analysis of oncogenic *ras* genes. Insertion mutations in oncogenic tyrosine kinases seem to be a particularly rich source of temperature-sensitive (ts) mutants.^{7,42} Some of these may be specifically defective in their interactions with key substrates at the nonpermissive temperature. The majority of ts mutants, however, lose both kinase and transforming activities at the nonpermissive temperatures. These latter mutants are valuable tools in following the time course of events in kinase-generated signals.

Insertions with no apparent effect on activity may indicate the position of hinge structures, or of regions that are irrelevant to the properties under examination. They can be useful starting points for the construction of chimeric proteins.³⁶

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