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MICROINJECTION STRATEGIES FOR THE STUDY OF MITOGENIC SIGNALING IN MAMMALIAN CELLS

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1. ABSTRACT

First used in the analysis of dynamic changes in cell structure, microneedle microinjection allows *in situ* study of individual living cells as opposed to large scale metabolic analysis of heterogeneous cell culture. In addition, microinjection also offers the possibility to examine *in vivo* regulated processes by modulating the intracellular levels and activity of key regulatory proteins and genes in both a specific and controlled manner. A number of different strategies have been developed over the past 5 years to examine the pathways and effectors that are involved in mitogenic signaling as well as in the regulation of gene expression during the proliferative response to growth factors by normal fibroblasts. These strategies include: 1. Direct *in vivo* competition for various trans-activating DNA binding activities by microinjection of double-stranded oligonucleotides, microinjection of monospecific antibodies against transcription factors and microinjection of dominant negative mutants of transcription factors based upon their DNA binding domain. 2. Microinjection of purified enzymes (kinases and phosphatases) or peptides and antibodies that specifically inhibit these activities. 3. Microinjection of expression plasmids which encode various normal and epitope-tagged regulatory molecules**.**

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In many of the experiments described below, c-*fos* gene expression was monitored as an early marker of mitogenic response. The c-*fos* gene belongs to a family of genes whose transcription is activated very early after addition of growth factor (1-4). For *in vivo* studies, the c-*fos* promoter offers several unique advantages. Primarily, it is easy to manipulate. In practical terms, when mammalian fibroblasts are made quiescent (by replacing the normal growth media, with growth factors-depleted media) and subsequently activated by re-adding mitogen (growth factors, serum), c-*fos* RNA expression is restored within 15 minutes and the protein is specifically detected in the nuclei of cells after 90 minutes, but is no longer detectable after 3 hours. Secondly, results obtained with the c-*fos* promoter are directly applicable to cell growth since expression of c-fos is itself a prerequisite for proliferation as demonstrated by microinjection of anti-fos antibodies which prevented proliferation in mammalian cells (5). Thirdly, the c-*fos* promoter is exquisitely sensitive to agents which cause cell stress. In this respect, heat-shock, poor microinjection or microinjection in the presence of heavy metals or

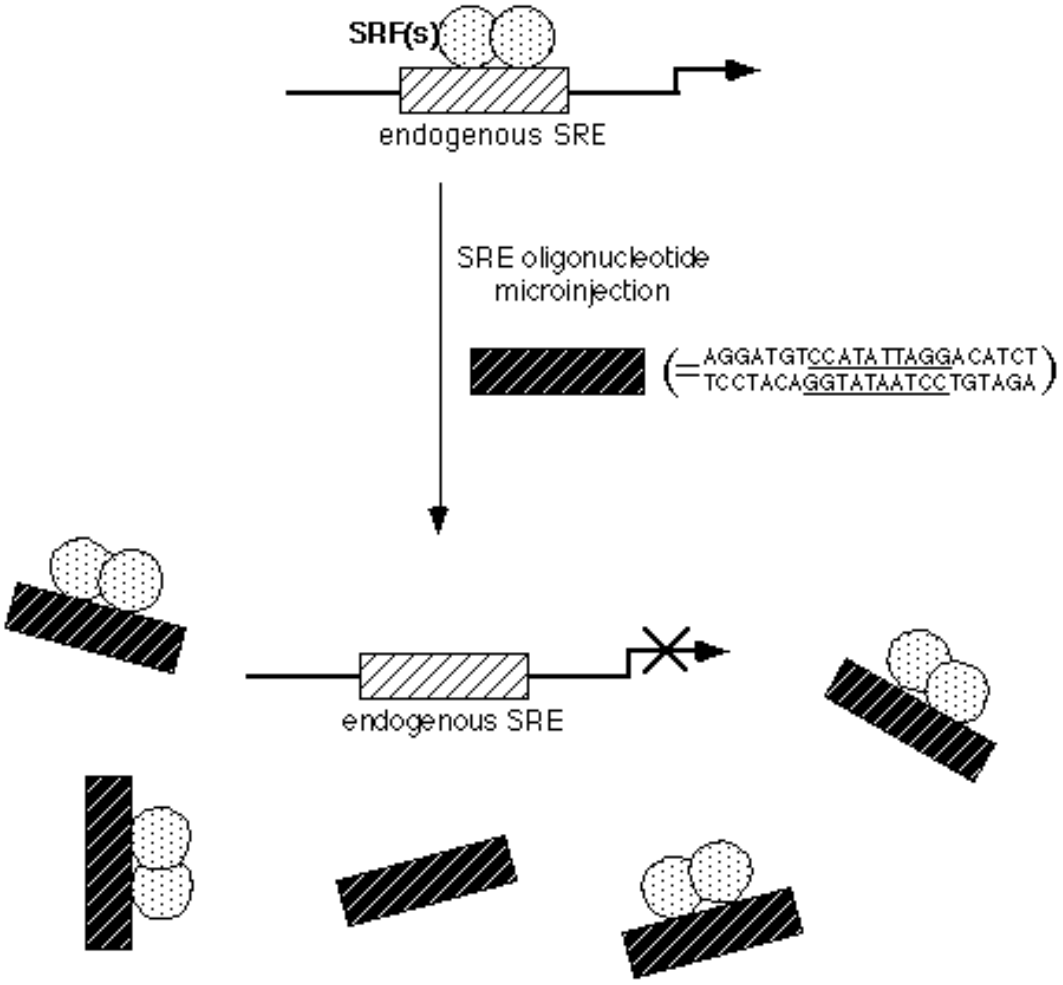

Fig.1: Schematic representation of *in vivo* competition through microinjection of synthetic SRE oligonucleotides.

chelating agents in the culture media all rapidly stimulate c-*fos* expression. However, when compared to *c-fos* expression in the proliferative response, stress mediated c-*fos* expression is induced both more rapidly and strongly, reverses more slowly (the protein is still detectable after 5-6 hours) and does not result in cell proliferation (unpublished observation). As such, it provides an excellent internal control for identifying poor treatment and manipulation of cells . Finally, the c-*fos* promoter is subject to several levels of auto-regulation enabling the analysis of not only components involved in transcriptional activation , but also various aspects of transcriptional down regulation and shut-off.

2.1 Oligonucleotide microinjection for *in vivo* competition in the study of DNA binding transcription factors

The current dogma is that transcriptional regulation of gene expression is mediated through DNA binding proteins, termed transcription factors. These factors, through their interaction with specific DNA sequences in the 5' regions of genes, modulate the capacity of RNA polymerases to transcribe the downstream 3' coding region.

The Serum Response Element (SRE) present in the promoter region of several genes including *c-fos*, requires the binding of SRF, in a dimeric form (together with other accessory proteins),

to activate expression of the gene downstream. Expression is prevented by competitive squelching of the SRF transcription factors, which bind to microinjected oligonucleotides corresponding to the sequence of the endogenous SRE. In the SRE sequence shown, the core consensus $CC(AT)_6GG$ (also named CArG box) is underlined.

In studies developed principally by Treisman and Gilman (7), it was clearly shown that the transcription factors bound to DNA in a sequence specific manner through precise DNA sequences ranging in size from 7 to 30 nucleotides (6,7).

To examine the role of transcription factors in mediating mitogenic or oncogene signaling *in vivo*, one strategy involves microinjecting synthetic double-stranded oligonucleotides containing a specific DNA-binding motif into the cytoplasm of cells. These small sequences rapidly migrate into the nucleus where they specifically compete with the endogenous sequences for the DNA binding activity of the related transcription factors (Fig. 1).

This technique was used in particular, to study the regulation of c-*fos* expression and subsequent cell proliferation. Of the major regulatory elements present in the 5' region of the c-*fos* promoter, the Serum Response Element (SRE), is a region of DNA 300 bp 5' of the transcription initiation site closely juxtapositioned to the TPA response element (TRE). SRE is known to bind the ubiquitous 67 kD mammalian transcription Serum Response Factor (SRF or p67SRF) (8-10), whereas the TRE binds Fos-Jun AP1 complex (reviewed in 11). The study of SRE is of particular interest since it may be implicated in transcriptional control of numerous serum responsive genes. *In vivo* competition studies were performed using synthetic double stranded oligonucleotides corresponding to the sequence of SRE [which contains the typical core sequence $CC(AT)_6GG$, also named CArG sequence], or as a control, a mutated oligonucleotide, in which essential oligonucleotide pairs required for interaction with SRF were changed. Using this approach, microinjection of SRE oligonucleotides into synchronized fibroblasts has allowed to show the direct implication of the SRE-containing region not only in c-*fos* induction following serum addition (12), but also in *c-fos* repression in quiescent cells together with another short DNA sequence, present in the coding region of *c-fos* (13). Using a similar strategy, the binding of protein factors at cAMP responsive elements (CRE), was shown to be necessary in mediating the induction of *c-fos* by cAMP (14).

2.2 Antibody microinjection for *in vivo* study of transcription factors activity

While the technique of oligonucleotide microinjection has been highly useful in the analysis of SRE/TRE regulated functions, it is restricted in its application. Oligonucleotides have short half lives *in vivo* and may present broad specificity by interfering with the binding of several proteins acting at the same promoter site. An alternative method of interfering with transcription factor activity involves microinjection of antibodies directed against the particular transcription factor. Antibodies offer the advantages of relatively long half lives *in vivo* and more restricted specificity since the antigenic epitope can be specifically targeted to a single protein (see table 1).

To complement the data obtained with oligonucleotides microinjection, the effect of microinjecting synchronized cells with monospecific mouse and rabbit polyclonal antibodies raised against different peptide sequences of the SRF protein outside its DNA binding domain, was examined. One limitation of immunoglobulin microinjection is that access to nuclear target proteins (such as SRF) requires direct microinjection into the nucleus (since Igs do not freely pass the nuclear membrane). However, nuclear injection is not applicable for the analysis of c-*fos* expression because it causes a stress sufficient to induce *c-fos* within 60 minutes after microinjection (unpublished observation). The problem can be overcome by cytoplasmic microinjection of the antibody several hours before mitogenic stimulation. The long half life of immunoglobulins allows effective neutralization of the newly synthesized cytoplasmic pool of nuclear proteins, resulting in a depletion of the nuclear pool of these proteins. This case has been illustrated for SRF, where a clear loss of endogenous nuclear SRF was shown to occur 7-8 hours after the cytoplasmic injection of antibodies against SRF (15). In addition, this approach allowed a clear estimation of the half-life of the protein, which is the time required for complete immuno-depletion of SRF from the nucleus. This method precludes the need for drugs such as cycloheximide which block overall protein synthesis and may yield an artifactually extended protein half life, particularly if the protein degradation requires the synthesis of a rapidly turned over protein.

The neutralization of SRF *in vivo* through microinjection of specific antibodies, has shown that activation of SRF (through binding and/or post-translational modification) is required for c-*fos* induction and subsequent entry into S phase in living cells (15,16).

Table 1: Microinjection tools for the *in vivo* study of transcription factors.

Tools	effects	Observations
Double stranded oligonucleotide	Competes with the endogenous promoter elements for DNA binding of proteins factors	- rapidly active (immediate diffusion) - short half-life (rapidly degraded) - limited specificity (interfere with all proteins that bind to the promoter element used)
monospecific neutralising antibody	Inhibitory by binding to its target antigen or inductionof nuclear depletion by cytoplasmic sequestration of newly synthesized proteins.	- highly specific and inhibitory antibodies are required - requires nuclear injection for an immediate effect or waiting for the turnover of the protein for nuclear depletion
DNA-binding region of the protein	Binds to the endogenous promoter element without activating transcription	- highly specific for the factor - not too rapid degradation - immediatly efficient (rapid nuclear diffusion and dynamic protein-DNA interaction) allows kinetic assay of an effect
Protein kinase or its specific inhibitor peptide	Raises or inhibits the activity of a specific kinase and the pathway downstream from it	-needs a pure and active kinase - availability of really specific inhibitory peptide for injection is restricted and its half-life is often short.
Coding or antisense plasmid	Raises or suppress the synthesis of a specific protein factor	- not applicable for kinetic studies of immediate effects - allows the use of tagged constructs to distinguish from endogenous expression - antisense injection can be specifically rescued by injection of the corresponding protein product

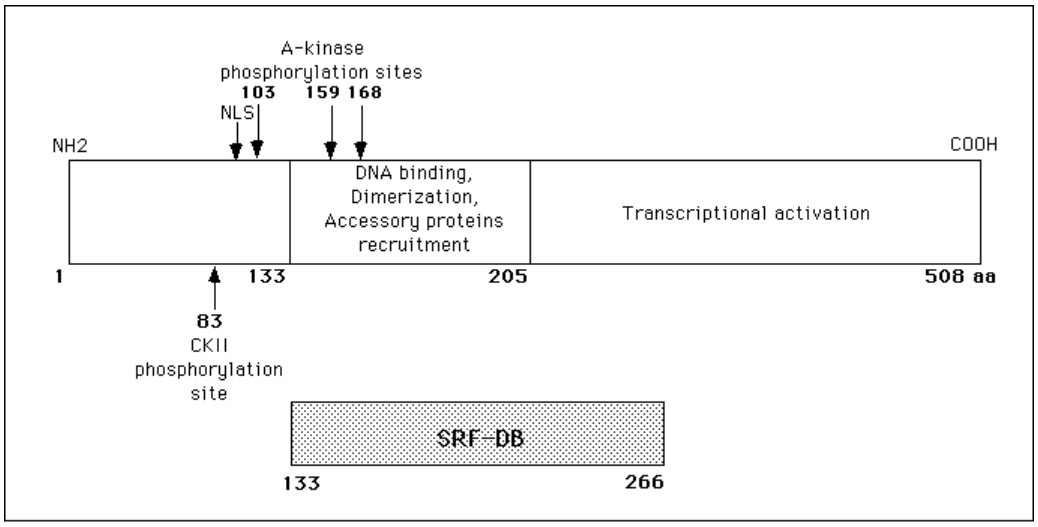

Figure 2: Schematic representation of the different domains and phosphorylation sites of human SRF protein. The presence of known consensus phosphorylation sites along the protein is indicated. The place of the recently identified (28) nuclear localization signal (NLS) is also marked, and the portion of SRF protein used as a dominant negative mutant of SRF is shown as a shaded box underneath.

Moreover, events occurring long after the initial stimulation by serum, also require SRF since DNA synthesis was blocked by microinjecting antibodies to SRF up to 8 hrs after serum addition.

A similar approach, exploiting the advantage of the long half life of microinjected antibodies, has been used to demonstrate the implication of SRF in skeletal muscle differentiation, a process which involves the fusion of myoblasts into myotubes over a period of 3 to 4 days. When injected into myoblasts, anti-SRF antibodies completely prevented the expression of early differentiation markers such as myogenin and subsequent fusion of myoblasts while a majority of surrounding uninjected cells expressed differentiation markers and underwent fusion into myotubes (17).

These examples illustrate the value of microinjection of antibodies to probe and validate *in vivo*, the role of a transcriptional factor such as SRF which positively regulates diverse processes such as c-*fos* gene induction, cell cycle progression to S-phase and myoblast differentiation.

2.3 Dominant negative mutant of transcription factors based upon their DNA binding domain

Since the use of antibodies *for microinjection* requires production of antisera reactive against native proteins, often a tedious process, a complementary strategy to interfere *in vivo* with transcription factors was developed. The approach consists of engineering a portion of the protein corresponding to its DNA binding domain devoid of transcriptional activation domains.

To test this approach for SRF, a portion of SRF spanning amino acids 113 to 265 containing the DNA binding domain, SRF-DB (see Figure 2), was expressed in bacteria, purified through DNA-affinity and microinjected into living cells.

Microinjection of SRF-DB effectively prevented c-*fos* expression and DNA synthesis stimulated by growth factors, two events described to require SRF, implying that an excess of SRF-DB injected into living cells can act as a dominant negative mutant (18). This strategy demonstrates the efficiency of a method for generating dominant negative mutants by using the DNA binding region of transcription factors and may allow the future study of events at the DNA/protein level. This approach has other advantages. First, it is not restricted to SRF but can be used in studying all transcription factors or similar DNA-binding proteins for which a defined DNA binding domain has been identified. Second, since the DNA binding sequences are unique for each transcription factor, this approach provides a sharp specificity for the probes. Third, the polypeptide encoding the DNA-binding domain is generally small enough to easily pass through the nuclear membrane and therefore does not require nuclear microinjection (see table 1). Fourth, this technique is insensitive to potential variations in antibody-antigen reactivity *in vivo* (which may arise with multimeric protein complexes). As such, this ensures that the effects observed are directly related to the injected sequence. Finally, it is possible to develop several DNA binding molecules containing different associated modules (for example dimerization or nuclear localisation

domains) to study the relative *in vivo* contribution of each of these individual domains.

3. METHODS FOR THE STUDY OF KINASES AND PHOSPHATASES

3.1 Microinjection of purified kinases and phosphatases to investigate the regulatory mechanisms involved in mitogenic activation

Entry into G_1 phase involves switching from quiescence (G_0) to a proliferative state, with the induction of "immediate-early" genes. Stimulation of these early genes does not necessarily require protein synthesis, implying that appropriate regulatory factors are present before growth factor stimulation (i. e. in G_0) (2,19). This is the case for SRF, which is present in quiescent cells, and after the inductive signal has been received, induces the expression of the early genes, such as c-*fos*. The rapidity of proliferative activation is due to the presence of such proteins even in G_0 phase, whose regulation is by virtue of post-translational modifications, and predominantly by phosphorylation. The potential role of different protein kinases or phosphatases activated by growth factors in the subsequent activation of transcription factors, has been studied by microinjection of different purified enzymes. In the case of c-*fos* gene expression, two kinases were studied : casein kinase II (CKII) and Ca^{+2}/phospholipid-dependent protein kinase (C-kinase) (15,20). Microinjection of either purified CKII or C-kinase resulted in the induction of c-*fos* in quiescent cells (with similar kinetics as serum induction). CKII is a kinase which is recruited to the nucleus at the G0/G1 transition. This enzyme has different nuclear targets, suggesting its implication in the final step of mitogenic signaling i.e. phosphorylation of transcription factors. Following CKII microinjection into quiescent cells, SRF was phosophorylated (15), and such phosphorylation was shown to slightly increase the DNA binding affinity of SRF and to markedly increase the rate of SRE-SRF exchange *in vitro* (21,22). Moreover, the possibility of coinjecting oligonucleotides corresponding to different protein binding DNA sequences together with protein kinases, has allowed identification of whether a promoter sequence is involved in the transcriptional activation by a specific kinase pathway. The co-injection of SRE oligonucleotide with CKII or C-kinase showed that activation at the SRE site (previously shown to be necessary for serum-induced c-*fos expression*) is also absolutely required for c-*fos* expression induced by CKII and C-kinase (table 2). Indeed, microinjection of SRE oligonucleotides with CKII or C-kinase totally inhibited c-*fos* expression clearly showing that positively acting factors activated by CKII and C-kinase must bind to the c-*fos* SRE to induce its expression. Immediately adjacent and 3' to SRE in the c-*fos* promoter is a short sequence corresponding to c-*fos* AP1 binding site also called TPA Responsive Element (TRE). Displacement of TRE binding factors by microinjection of TRE oligonucleotides resulted in a delayed induction of *c-fos* by serum and markedly prolonged expression of c-*fos* induced by serum or CKII indicating that TRE could be involved in the down-regulation of c-*fos* expression (20). However, TRE microinjection blocked c-*fos* induction by C-kinase or by ras oncogenic protein, suggesting that the induction of *c-fos* by these two effectors utilizes a pathway more restrictive and different from the pathway of induction by SRF or serum.(20) (table 2). In a similar manner, microinjection of protein phosphatases was used to probe their role in the activation or down-regulation of specific mitogenic pathways. In particular, using cells transfected with a reporter LacZ gene under the control of a TRE (recognized by the transcription factor complex AP-1), Alberts *et al.* (23) have shown that microinjection of purified protein phosphatase type 2A (PP2A) enhanced β-galactosidase expression after serum stimulation, whereas microinjection of phosphatase type 1 (PP1) did not . This effect was correlated with the dephosphorylation of negative regulatory sites on c-Jun, *in vitro* (23). Inversely, microinjection of PP2A had no effect on cAMP-induced expression of a reporter gene carrying a cAMP Responsive Element (CRE), whereas PP1 injection abolished that expression (23). In these examples, microinjection shown different regulatory roles for the two major phosphatases, in expression control by distinct mitogenic pathways.

This approach allows to rapidly and transiently increase the level of a kinase or phosphatase and follow the effect of this elevation either on substrate phosphorylation or on a particular physiologic process such as c-*fos* gene expression, cell shape modification, etc.). By direct injection of purified enzymes, one can specifically study the end result of activating a particular enzyme and its downstream effectors (see summary in table 1).

3.2 Microinjection of peptides and proteins inhibitory to kinases and phosphatases.

A complementary approach involves microinjection of a specific inhibitory peptide against kinases or phosphatases. This can be applied to studying the role of several different protein kinases (C-kinase, A-kinase, CKII), or phosphatases such as PP1. It may prove useful in situations where no inhibitory antibody is available. These peptides are synthesized based on the sequence of an endogenous inhibitory protein (for example PKI is a synthetic peptide analogous in sequence to a 20 amino acid proteolytic cleavage fragment which retains a high inhibitory activity for A-kinase and can be modified

Table 2: Effect of oligonucleotide microinjection on the expression and down-regulation of c-fos induced by serum, CKII, C-kinase and ras microinjection.

inducer of c-Fos	Time effect observed	no oligonucleotides	+SRE	+TRE/FAP
serum addition	90 min	+	−	−
	5 hrs	−	−	+
CKII microinjection	90 min	+	−	+
	5 hrs	−	−	+
C-kinase microinjection	90 min	+	−	−
	5 hrs	−	−	−
oncogenic Ras microinjection	90 min	+	−	−
	5 hrs	−	−	−

− : less than 5% of microinjected cells show *c-fos* expression

+ : 80 to 100% of microinjected cells show *c-fos* expression

to increase its stability *in vivo* (24)). The peptide can be synthesized by inserting a pseudosubstrate site in the regulatory domain from a given protein. this strategy has been used in synthesizing C-PKI, a synthetic peptide which acts as a potent C-kinase substrate antagonist (25)). These two peptides are suitable as probes in living cells. In particular, microinjection of the C-PKI peptide has been used to show the requirement of a C-kinase activity for *ras*-induced c-*fos* expression (12). A-kinase inhibition by PKI inside living cells resulted in marked effects on chromatin structure and cytoskeletal organisation (26) resembling those that accompany mitotic entry. Indeed, A-kinase appears to be a specific antagonist of many mitotic pathways (26,27). Such a global loss of A-kinase activity which prevents basic cell functions, prevents probing specific aspects of the cell metabolism. For example microinjection of PKI results in the exclusion of most transcription factors from the nucleus. Indeed, using microinjection of PKI, it was recently demonstrated that A-kinase activity is required in the process of active nuclear import of all proteins, including SRF (28). Another recent study addressing the role of protein phosphatases, used microinjection of a plasmid coding for a constitutively active form of inhibitor 1, a specific inhibitor of protein phosphatase type 1 (PP1), (29). These experiments proved that the transcription factor (CREB) that binds and activates the cAMP Response Element (CRE) is dephosphorylated on its A-kinase site by PP1, thereby limiting the transcriptional activity of CREB (29). This example combines the strategy of applying inhibitory peptides to specifically inactivate an enzyme pathway, and the use of expression plasmid microinjection, which allows the sustained overexpression of a peptide or a protein, as detailed below.

4. MICROINJECTION OF EPITOPE-TAGGED OR NORMAL EXPRESSION PLASMIDS.

4.1 Microinjection of epitope-tagged or normal expression plasmids

One of the principal difficulties in manipulating intracellular metabolism stems from the need for purified active components in a form

suitable for microinjection. An alternative strategy to microinjecting purified protein components, involves directly overexpressing cellular components through microinjection of DNA sequences in expression vectors under the control of efficient mammalian promoters. Two different types of plasmid DNA can be used to modulate cellular protein levels in either an additive or substractive manner: coding DNA constructs placed in a sense orientation after the promoter can be used to induce rapid elevation in the intracellular level of a given protein, whereas an antisense orientated DNA sequence will suppress the expression of a given protein.

4.1.1 Plasmid microinjection to overexpress proteins

As with most microinjection tools, the use of expression plasmids has both advantages and disadvantages (table 1). The major advantage of plasmid expression vectors is the production of soluble, homogenous, correctly folded and post translationally modified proteins within cells. This precludes the purification and isolation of proteins, which often leads to partial degradation and modification or inactivation of the protein. Finally, it is possible to couple genes of interest to short peptide tags which subsequently allows easy identification of the overexpressed protein within cells. Short epitope tags such as the hemagglutinin (HA) tag derived from influenza viral proteins can be particularly useful to determine the cellular localization of the overexpressed proteins by immunocytochemistry using anti-HA antibodies.

Whereas, in principle there is no difference between plasmid transfection and microinjection, the latter offers a number of advantages. Primarily, the efficiency of "transfection" by microinjection is close to 100% of the injected cells and therefore does not require any selection process. Secondly, the number of copies of the plasmid injected per cell can be accurately manipulated since the concentration of plasmid DNA in the needle is known, . Thirdly, different plasmids can be microinjected in close proximity on the same dish, allowing direct and simultaneous comparison of their effects under the same experimental conditions.

Three types of eukaryotic expression vector can be used. The first class is derived from eukaryotic viruses and contains deregulated highly efficient promoter sequences that lead to continuous and uncontrolled expression of DNA sequences placed downstream from the promoter region. The second class, termed inducible, contain promoters derived from various existing mammalian promoter sequences that respond to the addition of heavy metal ions or glucocorticoids. The third class comprise plasmids in which a specific promoter sequence from a known mammalian gene such as the *c-fos* promoter, is placed 5' from the gene of interest. These promoters respond in a manner identical to that of the normal gene which they regulate, leading to the expression of the protein of interest instead of Fos. To date, the first class of vector has been the most commonly used for specific overexpression of proteins. Within this group, two promoters have been predominantly used: the first derived from the polyoma SV40 virus and the other derived from the cytomegalovirus. Both classes of promoter are sensitive to the presence of growth factors, an important point to bear in mind when using them to study early events in cell activation.
There are some other limitations in the use of such plasmids for microinjection. First, they produce unregulated expression of the target protein, which within 2 to 3 hours of injection may lead to levels of protein in the cell that are far beyond physiological levels. As a result, any effect(s) and/or change in localization may in such cases, be artifactual. Second, since there is a lag time of 3-4 hours between microinjection and expression, plasmid injection is inappropriate for studies of highly dynamic intracellular processes.

Plasmid microinjection has been used to study the consequences of overexpressing dominant negative forms of the ubiquitous mitogen activated kinase (MAP-kinase). This kinase is activated by a serial cascade initiated at the membrane by activation of receptor tyrosine kinases. This results in a signal to MAP kinase which gets phosphorylated on tyrosine and threonine residues and subsequently becomes activated (30). Two expression vectors were used, encoding either wild type MAP kinase or a mutant form in which essential tyrosine and threonine residues had been eliminated by mutation to phenylalanine and alanine. These gene constructs were placed downstream of a CMV promoter and subsequently microinjected into rat fibroblasts. Cells were microinjected with the expression plasmids whilst still proliferating and then placed in serum free medium for 24 hours to shut down the mitogen activated pathways. The reinitiation of proliferation upon serum addition was then assessed by measuring passage through S-phase. The level of expression of MAP kinase was probed by immunofluorescence using either antibodies against MAP kinase or antibodies directed against the human influenza HA tag (monoclonal 12CA5). The simultaneous immuno-detection of BrdU incorporation (5bromo-deoxyuridine, an analogue of thymidine which incorporates into newly synthesized DNA when added to the culture medium), allowed determination as to which cells exhibited DNA synthesis. As anticipated, complete abolition of the proliferative response was observed in cells microinjected with the MAP kinase mutant plasmid. However, a similar inhibition was seen with both the wild type plasmid

and the vector alone. This point illustrates a possible pitfall in microinjecting plasmid DNA, particularly using the CMV vector system: an excess of vector alone frequently leads to an inhibition of cell proliferation. The reason is unclear, but is dependent upon the plasmid size, expression efficiency and the nature of the promoter region. SV40 driven expression vector plasmids produced a similar response although with less inhibition on cell proliferation when more than 20-30 copies of the plasmid were injected per cell. However, careful titration analysis using lower concentration of the CMV-driven plasmid showed that, when down to 2 copies of plasmid were injected into the nucleus of each cell, both the plasmid vector and the wild type kinase plasmid inhibited DNA synthesis in at least 80% of the cells. Whereas cells injected in their nuclei expressed the plasmid much more efficiently than when injected into the cytoplasm (in comparison to nuclear injection which yielded a consistent 85 - 95% expression, cytoplasmic microinjection yielded no more than 30% expression of the protein), none of the cells injected with control plasmids in the cytoplasm were inhibited in their passage through DNA synthesis. Therefore, in these experimental conditions, only cytoplasmic injection allows to identify a specific inhibition of cell proliferation by the dominant negative mutant of MAP kinase.

These data illustrate one of the most important points when using plasmid microinjection (and microinjection in general): the control for an experiment involving microinjection must always be very careful and well thought through. Such control is a necessary key to the interpretation of any microinjection result. In addition, for the study of protein kinases and phosphatases, plasmid injection strategy should be confronted, when possible, with injection of purified active proteins because cells may counteract the plasmid mediated overexpression as it occurs by overexpressing a corresponding regulatory or inhibitory subunit. Transfection approaches are even more prone to such problems because they require longer time scale experiments. In contrast, injection of highly purified, highly active kinases and phosphatases temporarily circumvent the entire cellular regulatory mechanism by completely skewing the intracellular metabolic balance for 1-2 hours.

4.1.2 Plasmid microinjection to produce antisense RNA

The use of microinjection in antisense experiments illustrates the perfect combination between microinjection, biochemistry and immunology. This technique was first used to demonstrate in a formal manner the absolute requirement of the protein cyclin A in DNA synthesis at a time when it was believed that this protein is uniquely involved in the mitotic activation (31). Microinjection of an SV40 expression vector encoding the full length anti sense cyclin A RNA into synchronized mammalian cells completely suppressed cyclin A expression at S-phase. This was shown immunofluorescence of cyclin A protein and simultaneous inhibition of DNA synthesis. This effect was exclusive and specific to cyclin A since antisense to the mitotic cyclin, cyclin B, did not produce a similar block. However, to provide formal proof that the effect observed with the injection of antisense RNA was exclusively related to depletion of cyclin A, cells were subsequently microinjected with purified cyclin A proteins. This specifically reversed the block to DNA synthesis. This technique of antisense rescue represents probably the most formal demonstration for a specific effect that can be provided by microinjection at the present time. In our hands, it is also possible to rescue cells injected with antibodies, but this involves the use of extremely high and unphysiological levels of antigen.

5 CONCLUDING REMARKS

As a whole, microinjection provides a powerful and unique tool to analyze the processes involved in cell activation and growth. Carefully controlled, it can be used to specifically interfere with transcriptional activation, post-transitional modification or the expression of particular proteins. In many cases, microinjection of specific inhibitors or activators facilitates the study of short term cellular events in a temporally controlled manner. Cellular processes can be shut-down, inhibited, prevented, activated or competed, in a direct way at precise and chosen moments. Through microinjection of different solutions on the same dish or coverslip, many potential artifacts can be controlled for in a single experiment, providing that a statistically valid number of cells can be injected with facility.

As the nature and complexity of cellular regulation grows continuously, the capacity to directly manipulate given targeted processes becomes more important. In allowing targeted manipulation of cell environment in the context of the living cell, microinjection provides a unique window on cellular events. Only by directly manipulating single cellular components, will it become possible to identify subtle nuances in the inter-relation between different proteins and cellular pathways. With the rapid advance of the identification of gene sequences encoding all human proteins, we will soon be at the point where many structural motifs, post-transitional modifications and protein interaction sites will also be identified. By allowing the direct manipulation of cellular processes, protein expression, activity and cytolocalization, microinjection should play an integral role in the dissection of the events involved in mitogenic activation. The uses we have described in this review represent some of the initial learning

steps that will pave the way to unveil the intricate processes involved in mitogenesis.

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