The ability of insulin to regulate the transcription of specific genes is an important facet of homoeostatic control in vivo. Insulin almost certainly regulates the activity of trans-acting factors which bind to specific nucleotide elements within the promoter regions of these genes. Interestingly, insulin can both positively (e.g. phosphoenolpyruvate carboxykinase) and negatively (e.g. glucokinase and fatty acid synthase) regulate the expression of genes [1]. Any plausible hypothesis for a molecular mechanism must take this into account.

The past 5 years have seen the identification of several distinct insulin-response sequences within a number of promoters. Furthermore, during this time period, there have also been dramatic advances in our understanding of intracellular signalling pathways. Despite this, very little is known about the signalling pathways that are responsible for regulating the activity of these trans-acting factors in response to insulin.

The binding of insulin to its receptor initiates the activation of multiple parallel intracellular signalling pathways [2]. A very early aspect of insulin signalling is the tyrosine phosphorylation not only of the receptor itself but...
also of several intracellular substrates such as insulin receptor substrate (IRS)-1, IRS-2 and Shc [3–5]. The resulting phosphotyrosines formed on these molecules act as docking sites for other proteins, possessing src-homology-2 (SH2) domains, which further transmit the insulin signal to the cell’s interior.

Interaction between IRS-1 and PtdIns 3-kinase leads to activation of this enzyme and the consequent production of PtdIns(3,4,5)P$_3$ in cells [6]. Wortmannin, which inhibits PtdIns 3-kinase at nanomolar concentrations [7], blocks the ability of insulin to activate p70^{S6k}, a protein kinase that phosphorylates ribosomal protein S6 subunits [7]. The activation of p70^{S6k} can also be specifically blocked by a soluble inhibitor, the immunosuppressant rapamycin [8].

The interaction of IRS-1 and Shc with Grb2 leads, via the guanine nucleotide exchange factor Sos, to the activation of Ras, a small GTP-binding protein [2]. This, in turn, promotes the activation of a protein kinase call Raf-1, which phosphorylates and activates mitogen-activated protein (MAP) kinase kinase [also known as extracellular–signal-regulated kinase (ERK) kinase] (MEK). MEK then phosphorylates and activates the MAP kinases, ERK1 and ERK2 [9]. This protein kinase cascade is stimulated by a huge variety of mitogens and other extracellular stimuli.

It is highly likely that insulin regulates gene transcription, at least in part, by altering the phosphorylation state of specific transcription factors. The stimulatory effect of insulin on Gene33 expression in H4IIE cells has been reported to be blocked by wortmannin but not rapamycin. This suggests a role for PtdIns 3-kinase but not the p70^{S6k} pathway [10]. The inhibitory effect of insulin on phosphoenolpyruvate carboxykinase gene expression has been reported to be both sensitive [11] and insensitive [10] to wortmannin, although both studies agreed that the p70^{S6k} was not involved. As wortmannin blocks MAP kinase activation by insulin in some cell types [12], a role for this protein kinase cascade in the regulation of the transcriptional events remains a possibility.

Studies on the signalling processes underlying insulin’s ability to regulate gene expression would be considerably aided by the development of an experimental system that allowed a wide variety of specific inhibitors to be introduced into cells (i.e. peptides, antibodies, antisense oligonucleotides as well as dominant-negative inhibitors). We have developed a method by which the activity of the firefly luciferase gene, when placed under the control of an insulin-responsive promoter, can be monitored in single living cells in real-time. As a model, we have utilized the relatively strong collagenase promoter which possesses a single phorbol 12-myristate 13-acetate-response element (TRE) [TGA(G/C)TCA] which acts as the binding site for the AP-1 complex [13]. This complex is composed of a heterodimer between the c-Fos and c-Jun transcription factors (see [14] for review).

**Visualizing insulin-stimulated AP-1 complex activity in single living cells**

The human collagenase promoter was cloned upstream of the firefly luciferase gene to give the plasmid pCol.Luc. We have previously shown that the effect of insulin on collagenase promoter activity is largely mediated via the TRE [15]. This plasmid was microinjected into the nucleus of single CHO.T cells which were then incubated for 16 h in the absence or presence of insulin. As shown in Figure 1, subsequent imaging of the cells in the presence of 1 mM luciferin using an intensified photon-counting charged couple device (CCD) camera (Hamamatsu Photonics) revealed a dramatic increase in luminescence of individual cells on insulin treatment. This camera possesses an intensifier composed of two ‘microchannel’ plates which, in simple terms, amplifies a single photon input into an approx. 10$^7$ electron output. The resulting electrons excite a phosphor screen and the light output of this screen is monitored using a standard CCD camera. The camera allows direct quantification of the number of photons emitted by the cells and revealed that insulin had an approx. 40-fold effect on luciferase expression. We have previously reported that the expression of luciferase can be monitored continuously in the simultaneous presence of insulin and luciferin, and that luciferase luminescence is first detectable at approx. 2 h after the addition of insulin to the cells [15].

Luciferase expression from this promoter could not easily be detected in single cells stably or transiently transfected with pCol.Luc, using a variety of transfection protocols (DEAE-Dextran, Transfectam, lipofectin, calcium phosphate or electroporation) (results not shown). This presumably reflects the higher plasmid copy number that can be achieved by direct microinjection. Although luciferase expression has previously
been monitored in intact cells, this was only when expressed under the control of strong viral promoters [16–18].

To date, we have found that the Hamamatsu-intensified CCD employed in this study is the most suitable camera for this type of imaging (i.e. it is of sufficient sensitivity and has a very high signal/noise ratio). Liquid-nitrogen-cooled CCD and single microchannel plate intensifiers do not give sufficient sensitivity or signal/noise ratio or low enough background to detect the very weak signals.

Our initial studies on the regulation of the collagenase promoter were performed using the pGL2 series of plasmids which are commercially available from Promega Corporation (Madison, WI, U.S.A.). More recently, we have found a dramatic increase in sensitivity when using the pGL3 series of luciferase vectors. In this vector the peroxisomal targeting signal on luciferase has been removed, a consensus Kozak initiation signal has been incorporated and the codon usage improved for mammalian expression. In CHO.T cells, when equivalent amounts of plasmid are used, the collagenase promoter within a pGL3 background gives approx. 10–20-fold more luciferase expression than when pGL2 is used (results not shown). This small adaptation therefore considerably improves the sensitivity of the technique, and may allow the use of less-sensitive imaging devices thus reducing costs.

We needed to visualize those cells that had been microinjected, and at the same time monitor their viability and correct for variations in the volume microinjected. With this in mind, CHO.T cells were co-microinjected with pCol.Luc and a second plasmid possessing the aequorin cDNA, with a mitochondrial targeting signal, under the control of the non-regulated cytomegalovirus (CMV) promoter (the pCMV.Aqm plasmid) [19]. After incubation of cells in the absence or presence of insulin for 16 h, the levels of aequorin and luciferase expression could be independently monitored within the same cell.

**Figure 1**

Insulin-stimulated gene expression in single CHO.T cells microinjected with the pCol.Luc plasmid

CHO.T cells were microinjected with 0.3 mg/ml plasmid pCol.Luc and incubated for 16 h in serum-free medium supplemented with or without 200 nM insulin. Bright-field images were collected for 10 s under low-level illumination (top). Luminescence images of the cells were collected for 15 min with the cells incubated in PBS plus 1 mM luciferin (bottom).
This involved first incubating the cells with coelenterazine (the cofactor for aequorin) for 5 h, and then stimulating with the purinergic agonist ATP. This promotes a substantial rise in mitochondrial Ca\textsuperscript{2+} which binds to aequorin and results in the production of a photon event which can be detected using the intensified CCD camera [15,20]. The effect on aequorin luminescence peaks at \( \approx 8 \text{ s} \) and returns to basal by 15 s [20]. As the resulting luminescence is so transient, the cells can then be incubated with luciferin and the level of luciferase expression determined independently. Thus a ratio of luciferase/aequorin luminescence level gives an estimate of the specific activity of the AP-1 complex within the individual cell (see [15] for more details).

Interestingly, in a small proportion (approx. 10\%) of cells, we found that luciferase expression occurred in the apparent absence of aequorin expression and vice versa (results not shown). This was despite the fact that the cells were injected with a premixed preparation of pCol.Luc and pCMV.Luc. This suggests, perhaps, that the collagenase and CMV promoters are active only at certain points within the cell cycle. Consistent with this hypothesis, we have previously reported considerable heterogeneity in the kinetics of activation of the collagenase promoter by insulin within individual CHO.T cells. This hypothesis requires further testing.

In conclusion, the model system described is particularly suitable for dissecting the molecular mechanism by which insulin activates AP-1 complex activity.

**Role of MAP kinases in insulin-stimulated AP-1 complex activity**

The MAP kinases, ERK1 and ERK2, are rapidly activated by insulin (and other growth factors) [9,21,22]. ERK1 and ERK2 are activated by MEK, a dual-specificity protein kinase that phosphorylates a threonine and tyrosine residue within the regulatory domain of ERK1 (T\textsuperscript{185}EY\textsuperscript{186}) and ERK2 (T\textsuperscript{185}EY\textsuperscript{186}). Phosphorylation of both residues is required for activation of the enzymes [22,23]. MEK is itself phosphorylated on two serine residues by Raf-1 resulting in its activation [24]. Substitution of the Raf-1 phosphorylation sites on MEK with alanines (MK\textsuperscript{185}T\textsuperscript{186}) yields a non-activatable protein kinase that expresses a dominant-negative phenotype, i.e. MEK\textsuperscript{185}T\textsuperscript{186} specifically blocks growth-factor activation of MAP kinase in a variety of cell types [25].

MAP kinase can be also inactivated by a family of dual-specificity Thr/Tyr-phosphatases. These include human CL100 [26], murine 3CH134 [27] and PAC-1 [28]. These dual-specificity MAP kinase phosphatases selectively dephosphorylate MAP kinase when overexpressed in cells [27,28]. In addition, an enzymatically inactive form of 3CH134 (with Cys-258 substituted with serine; 3CH134\textsuperscript{258S}) may also interfere with normal ERK1/2 function by forming a tight complex with phosphorylated MAP kinase [27].

We have previously reported that the overexpression of CL100 in CHO.T cells, using microinjection, blocks insulin-stimulated AP-1 complex activity by \( \approx 75\% \) (Figure 2) [15]. The phosphatase-inactive CL100\textsuperscript{258S} mutant had a quantitatively similar effect on insulin-stimulated AP-1 activity to that observed with the wild-type CL100 [15]. As CL100 overexpression could lead to ectopic dephosphorylation of proteins unrelated to MAP kinases, we also examined the effect of overexpression of dominant-negative...
MEK (MEK^{S217/221A}). Overexpression of MEK^{S217/221A}, but not of wild-type MEK, blocked insulin-stimulated AP-1 activation (Figure 2). Finally, we also showed that a constitutively active form of MEK (MEK^{S217/221A}) promoted an insulin-dependent activation of AP-1 activity to the level observed with insulin alone (Figure 2).

It was important to confirm the data obtained in single cells with experiments carried out by transient transfection of cell populations. CHO.T cells were co-transfected with pCol.Luc and pSV.CAT [contains the chloramphenicol acetyltransferase (CAT) gene under the control of the Simian virus 40 promoter], and then incubated in the presence or absence of insulin. After 16 h the cells were extracted and the levels of luciferase and CAT were measured by luminometry and ELISA respectively. As shown in Figure 3, insulin stimulated AP-1 activity (as determined by the ratio of luciferase/CAT activity) by approx. 40-fold. Overexpression of wild-type CL100, mutant CL100^{C258S}, MEK^{S217/221A} but not wild-type MEK blocked insulin-stimulated AP-1 activity.

Using both microinjection and transient transfection approaches, we have found that the efficiency of inhibition of insulin-stimulated AP-1 activity is greatest with wild-type CL100 > CL100^{C258S} > MEK^{S217/221A}. This most likely arises both from the nature of inhibition and the level of expression of the inhibitory molecule. For example, wild-type CL100 will act enzymatically and thus would be expected to be the most efficient inhibitor. On the other hand CL100^{C258S} and MEK^{S217/221A} presumably act through the stoichiometric association with their target (i.e. MAP kinase in the former case, and Raf-1 and/or MAP kinase in the case of the latter).

Overexpression of a constitutively active form of MEK (MEK^{S217/221A}) caused an increase in AP-1 activity which closely approximated to that resulting from insulin treatment alone (Figure 3).

**Discussion**

We have developed a non-invasive and quantitative assay for monitoring the regulation of gene transcription by insulin in single living cells. This technique can be used to monitor insulin-stimulated gene expression in real-time.

The method allows the simultaneous detection of luciferase and aequorin when co-expressed within single living cells. This allows changes in the activity of a regulatable promoter to be normalized to that of a constitutive promoter (Figure 2), in a manner similar to that using a transient transfection approach (Figure 3). Previous methods of detecting reporter proteins in fixed and permeabilized single cells using immunofluorescence staining are largely non-quantitative and do not easily allow the dynamics of reporter gene expression to be followed.

The microinjection approach will allow the introduction of an almost unlimited array of molecular species (DNA, RNA, peptides, phospholipids, metal ions etc.) into cells. The species may be designed specifically to modulate the activity of a particular signalling component within a pathway. This should allow us to build up a detailed picture of the role of certain signalling pathways in the actions of a variety of extracellular stimuli on gene transcription. The technique can be adapted for looking at any promoter element as long as that promoter is sufficiently strong to allow luciferase detection. The technique is also well suited to a real-time *in vivo* analysis of other phenomena such as transcriptional regulation during the cell cycle as well as DNA–protein interactions and mRNA translation using microinjected mRNAs containing the
Luciferase-coding sequence.

We have used this approach to investigate the role of MAP kinase in insulin signalling to the nucleus in living mammalian cells. To do this we blocked signalling by MAP kinase by (i) inactivating ERK1 and ERK2 through the over-expression of the dual-specificity MAP kinase phosphatase CL100, (ii) sequestering ERK1 and ERK2 using the mutant CL100\(^{227/228}\), or (iii) preventing the activation of ERK1 and ERK2 using a dominant-negative MEK (MEK\(^{5217/5221}\)). All three interventions blocked the activation of the AP-1 complex by insulin. The results obtained in microinjection experiments (Figure 2) were almost indistinguishable from those obtained in experiments performed by standard co-transfection (Figure 3).

The precise mechanism by which MAP kinase regulates AP-1 complex activity in vivo is not known. The inhibition of insulin-stimulated AP-1 complex activity by CL100 is most simply explained by the requirement for activated ERK1/2 in the phosphorylation of the transcription factor p62\(^{2F4}\) which binds to and activates the c-Fos promoter in concert with the serum response factor [9]. Indeed, we have found that CL100, CL100\(^{227/228}\) and MEK\(^{5217/5221}\) all block the ability of insulin to stimulate luciferase expression from a c-Fos promoter (results not shown). Insulin has been shown to increase c-Fos mRNA levels in these cells [29] and this would lead to an increased mass of the AP-1 complex.

ERK1 and ERK2 may also phosphorylate the N-terminal (Ser-63 and -73) domain of c-Jun leading to its transcriptional activation [30]. However, it is debatable whether this occurs in intact cells where the stress-activated MAP kinases, JNK and SAPK [31,32], have been proposed to be the more relevant c-Jun (Ser-63 and -73) protein kinases [33]. This possibility requires direct testing, as JNK activity is not stimulated by insulin in CHO.T cells [15].

Luciferase imaging, coupled with a microinjection approach, provides the only currently available method that will allow the quantitative analysis of weak insulin-regulated promoter activities in single living cells. We have used this method to provide the first direct evidence in favour of a role for MAP kinase in insulin signalling to the AP-1 complex. This approach will be particularly suitable for studying insulin signalling in primary cultured cells, especially those that are available only in low abundance (e.g. from biopsy samples).

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Regulation of selenoprotein gene expression and thyroid hormone metabolism

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Introduction

Adequate dietary intakes of selenium are essential for normal health in man and animals. When insufficient selenium is available, a wide range of clinical conditions and diseases can occur, consistent with several biochemical functions of the trace element [1]. These functions are associated with selenoproteins, estimated to number between 20 and 100 in mammalian cells. In vivo labelling of rats with $^{75}$Se and subsequent separation of proteins by SDS/PAGE under reducing conditions has detected 20–30 selenoproteins [2,3]: reduction and boiling of proteins before electrophoresis removes selenium that is non-specifically bound but not that bound as the amino acid selenocysteine. In addition, Burk and Hill [3] have estimated that, since there are 10 minor proteins for each abundant protein in the genome and since 10–15 abundant selenoproteins have been discovered, the existence of 100 selenoproteins is not unreasonable. Most known selenoenzymes probably use selenocysteine as a redox catalyst since at physiological pH, the amino acid –SeH group is more likely to be ionized than an equivalent –SH group. This allows selenium to be a more active catalyst than sulphur. Cytosolic glutathione peroxidase, the first selenoprotein to be identified, was originally thought to have a ‘hyperactive sulphur’ at the active site [4]. As well as in selenoproteins, selenium has also been detected in some bases in tRNAs from both prokaryotes and eukaryotes; however, the biological function of this selenium is not known [5].

Selenium function is regulated through selenoprotein gene expression and this can be controlled in two important ways: by the availability of dietary selenium for incorporation into selenocysteine and by metabolic changes that influence biochemical pathways in which selenoproteins play a crucial role. For example, factors such as oxidative stress can increase selenium-containing glutathione peroxidase activities, and hyperthyroidism will decrease selenium-contain-