REVIEW ARTICLE

Nuclear protein phosphorylation and growth control

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INTRODUCTION

Protein phosphorylation regulates a diverse range of cellular processes. A rapidly growing area of interest in the study of protein phosphorylation is the regulation of cellular growth through phosphorylation of key nuclear proteins. The growing list of such nuclear 'effector' proteins includes proto-oncogene products that function as transcription factors as well as tumour suppressor proteins that may be involved in regulating the onset of DNA replication. Regulation of the activities of these proteins can occur through phosphorylation and dephosphorylation mechanisms that in turn respond to the timing mechanism of the cell cycle, or directly to extracellular signals transduced to the nucleus by 'cascade events'. In addition, some viral proteins that stimulate cellular growth are also regulated by phosphorylation or are able to influence the phosphorylation of proteins central to growth regulation (Fig. 1).

This Review examines the phosphorylation of a selected number of nuclear proteins that are involved in the control of cellular growth and that illustrate the extent of present knowledge about nuclear signal transduction. These proteins include the transcription factors, CREB, Jun, Fos, NF κ B, Myc and Myb, four of which are the products of proto-oncogenes (Jun, Fos, Myc and Myb); two tumour suppressor proteins, p53 and the product of the retinoblastoma susceptibility gene (pRB); and the simian virus 40 (SV40) large tumour antigen (T antigen) which is a multifunctional viral protein involved in DNA replication, transcription and cellular transformation. The effects of phosphorylation on biochemical activity and biological function are discussed, together with the protein kinases and phosphatases involved and their response to a variety of growth factors and tumour promoters. Interestingly, several of these proteins are phosphorylated by the same protein kinases, suggesting that signals may co-ordinately target these key proteins. In some cases, there is a relationship between nuclear phosphorylation events and cellular transformation in which oncogene products play a role. Each protein is *multiply* phosphorylated and in some cases phosphorylation at a particular site occurs in response to different signals. Finally, some proteins respond directly to external stimuli while others are phosphorylated temporally in response to the timing mechanism of the cell cycle. Taken together, these examples give an overview of the role of phosphorylation of nuclear proteins in growth control.

CREB

The cyclic AMP (cAMP) responsive element binding protein (CREB) mediates the stimulation of gene expression in response to factors that elevate intracellular cAMP levels (for detailed reviews see Montminy *et al.*, 1990, 1991; Bohmann, 1990). The identification of this protein established a direct link between the hormonal stimulation of cells and the modulation of transcriptional activity. CREB has also proved to be an excellent

example of a nuclear protein which mediates 'cross-talk' between different signal transducing pathways.

CREB was discovered (Montminy & Bilezikjian, 1987; Yamamoto *et al.*, 1988) through its interaction with the cAMP responsive element, a short palindromic core sequence (5'-TGACGTCA-3'; Montminy *et al.*, 1986; Comb *et al.*, 1986; Short *et al.*, 1986) which is present in cAMP-inducible promoters and which mediates cAMP-dependent stimulation of transcription (several of these promoters are described by Montminy *et al.*, 1991). CREB is a dimeric protein of subunit M_r 43000 (Hoeffler *et al.*, 1988; Gonzalez *et al.*, 1989) which contains three major structural elements: a DNA-binding domain rich in basic amino acids, a leucine zipper responsible for dimerization and a

Table 1. List of abbreviations

Abbreviation	Full name
AP-1	activator protein-1
CaMPK	calcium/calmodulin dependent protein kinase
cdk1	cyclin-dependent protein kinase 1 (synonymous with p34 ^{cdc2})
CKI	casein kinase I
CKII	casein kinase II
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
dsDNA-PK	double stranded DNA-activated protein kinase
EGF	epidermal growth factor
ERK*	extracellular signal-regulated kinase
ERT kinase*	epidermal growth factor receptor threonine kinase
GSK3	glycogen synthase kinase 3
HRI	haem-regulated eIF2 kinase
I κ B	inhibitory subunit of nuclear factor κ B
MAP*	mitogen-activated protein (kinase)
MAP2*	microtubule associated protein 2
MRE	Myb response element
NF- κ B	nuclear factor κ B
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C
PP2A	protein phosphatase 2A
pRB	the retinoblastoma susceptibility gene product
p34 ^{cdc2}	protein kinase encoded by the (cell division cycle) cdc2 gene (synonymous with cdk1)
RP-C	replication protein C
SV40	simian virus 40
T antigen	the large tumour antigen of SV40
t antigen	the small tumour antigen of SV40
TGF β 1	transforming growth factor β 1
TPA	12-O-tetradecanoylphorbol 13-acetate
TRE	TPA-responsive element
TSE1	tissue specific extinguisher 1

* Mitogen-activated protein kinases (MAP kinases), microtubule associated protein-2 kinase (MAP2 kinase), myelin basic protein kinase (MBP kinase), epidermal growth factor receptor threonine (ERT) kinase and extracellular signal-regulated protein kinases (ERKs) are all synonyms for the same family of protein kinases.

Abbreviations used are defined in Table 1.

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Fig. 1. Nuclear protein phosphorylation and growth control

Signals initiated at the cell membrane are transduced (via second messengers, kinases and/or phosphatases) to the nucleus. There, they converge on key nuclear proteins which are important for regulating growth. Other signals (e.g. arising from the timing mechanism of the cell cycle) may modulate protein phosphorylation. In addition, oncogene products may have potent effects on the phosphorylation of nuclear proteins. The symbol of a circle with the letter P in the centre (in this and in all other figures) signifies a phosphorylation site.

highly acidic transactivation region (amino acids 88–160) which contains a cluster of potential phosphorylation sites [for cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC), glycogen synthase kinase 3 (GSK3), casein kinase I (CKI) and casein kinase II (CKII); see Fig. 2]. These phosphorylation sites lie within a subdomain termed the P-box which is important for the induction of gene expression. CREB is now known to be identical to activating transcription factor 47 (ATF47) which is a member of a larger family of transcription factors (Hurst *et al.*, 1990). It is also closely related to the AP-1 family of transcription factors and its target sequence differs from the consensus AP-1 motif [also known as the TPA-responsive element (TRE)] in only one base (Hoeffler *et al.*, 1988; Gonzalez *et al.*, 1989).

CREB is phosphorylated by cAMP-dependent protein kinase (PKA) *in vitro* at Ser-133 (Yamamoto *et al.*, 1988) which lies within the P-box region of the transactivation domain. This residue is also phosphorylated *in vivo* in response to forskolin, an activator of adenylate cyclase (Gonzalez & Montminy, 1989). Phosphorylation by PKA has no effect on the ability of CREB to bind to the CRE, but enhances transcription efficacy *in vitro* by 20-fold (Yamamoto *et al.*, 1988). Mutational studies have shown that CREB is unable to activate transcription *in vivo* when Ser-133 is replaced by non-phosphorylatable amino acids (Gonzalez & Montminy, 1989; Lee *et al.*, 1990). Based on these findings, a model was proposed for the cAMP-dependent induction of gene expression. Following hormonal stimulation, increased cAMP levels promote dissociation of PKA holoenzyme. The active catalytic subunit is then transported to the nucleus (Nigg *et al.*, 1985) where it phosphorylates CREB at Ser-133. This phosphorylation event is thought to activate CREB by inducing a conformational change in the protein (Gonzalez & Montminy, 1989) which allows the negatively charged transactivation domain to interact with its target protein, the TATA-binding factor TFIID (Horikoshi *et al.*, 1988).

Two other regions within the transactivation domain are now known to be important for the stimulation of transcription by CREB. The first of these is the α peptide (Yamamoto *et al.*, 1990) which comprises amino acids 88–101. This α -helical region is encoded by an alternative exon and is absent from Δ CREB (alternatively designated CREB-327), a lower activity form of CREB. The α peptide interacts co-operatively with the PKA phosphorylation site and is important for maximal cAMP-dependent induction (Yamamoto *et al.*, 1990). The second subdomain, which is required for full transcriptional activity, lies within the P-box and is termed the PDE1 region. This domain was identified by deletion mapping and comprises amino acids 106–124 in the full-length CREB protein (Lee *et al.*, 1990; see Fig. 2). Multiple phosphorylation of PDE1 *in vivo* occurs independently of phosphorylation at Ser-133. CKII will phosphorylate all five serine residues within this peptide *in vitro* (Lee *et al.*, 1990), although the spacing of the five serine residues would also suggest that this region is a substrate for processive phosphorylation by CKI. It is not known which enzyme phosphorylates these residues physiologically, nor what effect phosphorylation of this region has on CREB activity. CKII is also able to phosphorylate the PDE3 region (amino acids 138–148), but loss of these residues does not appear to influence cAMP inducibility (Lee *et al.*, 1990). [CKII is a ubiquitous cyclic nucleotide-independent protein kinase so called because conventionally it has been assayed using casein as its substrate (for reviews see Pinna, 1990; Meisner & Czech, 1991). Considerable interest in CKII has arisen in the past few years because it has been reported to be activated in response to mitogens including serum (Carroll & Marshak, 1989), the tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA; Carroll *et al.*, 1988), insulin (Sommercorn *et al.*, 1987; Klarlund & Czech, 1988), insulin-like growth factor I (Klarlund & Czech, 1988) and epidermal growth factor (Sommercorn *et al.*, 1987; Ackerman &

Fig. 2. Phosphorylation of CREB

The CREB protein is shown schematically with the DNA binding domain/leucine zipper and the transactivation domain (containing the α peptide and P-box regions) highlighted. The numbers refer to amino acid positions in this 341-amino-acid protein. The sequence of the phosphorylated region (P-box) is shown together with those sites of phosphorylation that have been identified. A question mark (?) signifies that, although a consensus recognition sequence for a kinase is present, there is not yet any experimental evidence for phosphorylation by the particular kinase. Similarly, a question mark signifies that it is not known whether the site is phosphorylated by a particular kinase *in vivo*.

Osheroff, 1989). CKII has a broad substrate specificity and targets many nuclear proteins which have been implicated in growth control, some of which are discussed below.]

CREB is phosphorylated by other known protein kinases *in vitro* and may therefore be a target for other signalling pathways. PKC phosphorylates Ser-133 (which is also phosphorylated by PKA) and two other as-yet-unidentified sites *in vitro* (Yamamoto *et al.*, 1988). Phosphorylation by PKC (presumably at these other sites) increases binding of CREB to the CRE and stimulates formation of CREB homodimers (Yamamoto *et al.*, 1988). However, it is not yet clear whether CREB and PKC interact *in vivo*. Evidence to date suggests that TPA treatment of cells does not elicit a CRE-dependent stimulation of gene expression (Lee *et al.*, 1990; Sheng *et al.*, 1991; Hoeffler *et al.*, 1989; Karmar *et al.*, 1991), suggesting that PKC does not play a role, at least directly, in CREB-dependent activation of transcription.

Ca^{2+} mediates gene induction in response to membrane depolarization of various neuronal cell lines and this inducibility has been mapped to CRE-like elements in different promoters (Sheng *et al.*, 1988; Sheng & Greenberg, 1990; Nguyen *et al.*, 1990). In addition, rapid phosphorylation of CREB at Ser-133 has been demonstrated in response to membrane stimulation (Sheng *et al.*, 1990) which further implicates CREB in transducing Ca^{2+} signals. Additional support for this idea comes from the observation that a GAL4-CREB fusion protein can mediate Ca^{2+} or cAMP inducibility of a reporter construct containing a GAL4 binding site only if the CREB contains serine at position 133 and not alanine (Sheng *et al.*, 1991), suggesting that phosphorylation of Ser-133 is essential for this effect. These findings show that CREB is responsive to both second messengers and that Ser-133 of CREB is a convergent point for two signal transducing pathways. Since TPA treatment does not induce CRE-mediated stimulation of transcription, it is likely that some Ca^{2+} -dependent protein kinase(s) other than PKC mediates the effect. Two candidate kinases are Ca^{2+} /calmodulin-dependent

protein kinases (CaMPK) I and II, both of which phosphorylate CREB at Ser-133 *in vitro* (Sheng *et al.*, 1991; Dash *et al.*, 1991). However, it is not yet known whether these kinases are the physiological mediators.

Further evidence of a role for CREB in cross-talk between signal transducing pathways comes from the effects of transforming growth factor β 1 (TGF β 1) on gene expression in mink lung cells (Kramer *et al.*, 1991). TGF β 1 mediates its effects through the TRE and not the CRE (de Groot & Kruijer, 1990) and does not affect the intracellular level of cAMP (Kramer *et al.*, 1991). However, TGF β 1 (but not TPA) does induce phosphorylation of CREB and significantly stimulates the ability of this protein (as part of a heterodimer with a Fos-related protein) to interact with the TRE (Kramer *et al.*, 1991). CREB is therefore a target for phosphorylation (at an, as yet, unidentified site) by an, as yet, undefined cAMP-independent and TPA-independent signal, yet participates in transcriptional stimulation through an element normally associated with TPA responsiveness.

One final interesting aspect of the role of CREB phosphorylation in growth control and differentiation comes from studies of the loss of tissue-specific gene expression which occurs when different cell types are fused (Boshart *et al.*, 1991; Jones *et al.*, 1991). This process of 'extinction' is thought to be mediated by *trans*-dominant regulatory factors. One such tissue-specific extinguisher of hepatocyte-specific genes, TSE1, represses transcription via the CRE and can be reversed by cAMP-dependent stimulation of transcription (Boshart *et al.*, 1991; Jones *et al.*, 1991, and references therein). TSE1 has recently been shown to encode the RI α regulatory subunit of PKA (Boshart *et al.*, 1991; Jones *et al.*, 1991). Increased tissue-specific expression of TSE1/RI α is therefore thought to sequester free catalytic subunit with the effect of reducing the basal level of PKA activity. This in turn leads to a reduction in the phosphorylation of CREB and a corresponding decrease in

transcriptional transactivation at promoters containing the CRE. These studies highlight a role for CREB in the tissue-specific expression of genes in addition to its established role in mediating transcriptional effects in response to external signals.

Jun AND Fos

The products of the *c-jun* and *c-fos* proto-oncogenes are members of the AP-1 (activator protein-1) family of transcription factors which mediate transcriptional stimulation through their interaction with a specific DNA sequence (TGAC/GTCA; reviewed in Abate & Curran, 1990; Woodgett, 1990; Karin, 1991a,b; Lewin, 1991). This element is known as the AP-1 site or, in some cases, the TPA-responsive element (TRE), because transcriptional transactivation by signals from the protein kinase C pathway occurs via this sequence. The functional form of AP-

1 is a dimer in which two Jun monomers or one Jun and one Fos monomer associate through interaction of leucine zipper motifs.

Jun was first observed as the product of the transduced oncogene *v-jun* in the avian sarcoma virus ASV-17 (Maki *et al.*, 1987). Its biochemical activity had already been strongly implied to be that of a transcription factor since it both bound the same DNA motif and showed structural similarity to the yeast transcriptional activator GCN4, particularly within the DNA binding region of this protein (Vogt *et al.*, 1987). Indeed the Jun and GCN4 DNA binding domains were shown to be functionally equivalent (Struhl, 1987). The similarity between *v-jun* and its cellular counterpart (in chicken) *c-jun* is about 80%; the differences are that *v-Jun* lacks an N-terminal region of 27 contiguous amino acids (termed the delta domain), and has two missense changes (D. Gillespie, personal communication) within its C-terminal region (there were originally thought to be three changes). The *v-Jun* protein is more highly transforming and a stronger transactivator than *c-Jun*, which has been attributed mainly to the absence from *v-Jun* of the delta domain (Fig. 3a; Baichwal & Tijan, 1990; Bos *et al.*, 1990).

Although there is a *direct* interaction between PKA and CREB in the cyclic AMP signalling pathway, there is no such direct interaction between PKC and *c-Jun* in the TPA-stimulated pathway. Rather, TPA treatment of cells induces rapid *dephosphorylation* of Jun at a specific site(s) which lies very close to the DNA binding domain (Boyle *et al.*, 1991). This region contains three residues, Thr-239 and Ser-243 and -249, which are phosphorylated *in vivo* and can be phosphorylated *in vitro* by glycogen synthase kinase 3 (GSK3; Boyle *et al.*, 1991). [GSK3 is a kinase which was originally associated with glycogen metabolism, but is now thought to play a major role in growth control and development (Woodgett, 1991).] Phosphorylation of a bacterially expressed Jun protein by GSK3 blocks the ability of Jun to interact with the TRE, suggesting that phosphorylation may regulate DNA binding *in vivo* (Boyle *et al.*, 1991). [Another kinase reported to phosphorylate this site *in vitro* is the ERT (epidermal growth factor receptor threonine) kinase (Northwood *et al.*, 1991), but it is not known which kinase phosphorylates these sites *in vivo*.] It is interesting that mutation of Ser-243 to phenylalanine (a change naturally occurring in the *v-Jun* protein) prevents phosphorylation of all three C-terminal sites *in vivo* and promotes AP-1 activity by about 10-fold (Boyle *et al.*, 1991). Loss of the ability to down-regulate Jun by phosphorylation of this region may therefore be part of the mechanism of oncogenic activation of Jun. Based on these studies, Boyle *et al.* (1991) have proposed that Jun is phosphorylated at the 'GSK3' sites in resting cells and is rapidly activated by dephosphorylation following stimulation of a protein phosphatase by the PKC pathway.

Activation of Jun in response to TPA treatment may also be mediated partly through changes in phosphorylation of residues closer to the N-terminus. Phorbol ester treatment also induces phosphorylation of two N-terminal serine residues at positions 62 and 72 (Pulverer *et al.*, 1991). Jun proteins in which these residues have been mutated to encode leucine residues have impaired transactivation activity (Pulverer *et al.*, 1991) suggesting the phosphorylation of these sites is required to activate Jun. Both of these residues lie within a proline-rich region required for transactivation and are substrates for phosphorylation *in vitro* by mitogen-activated protein kinases [Pulverer *et al.*, 1991; mitogen activated protein (MAP) kinases are a family of serine/threonine kinases which play a central role in signal transduction and are described briefly below]. The finding that a TPA-induced Jun kinase co-purifies from cell extracts with the pp42/44 and pp54 MAP kinases, and can be inhibited by incubation with a tyrosine phosphatase, suggests that Jun is

Fig. 3. Phosphorylation of the Jun and Fos proteins

The *c-Jun* (panel a) and *c-Fos* (panel b) proteins are shown schematically. The positions of functional and structural domains are indicated. Numbering refers to amino acid residues. Regions containing phosphorylation sites are expanded and the amino acid sequences given, together with the known enzymes which mediate the phosphorylation and dephosphorylation reactions.

phosphorylated by MAP kinase(s) *in vivo* (Pulverer *et al.*, 1991). It is interesting that the Ha-*ras* oncogene, which has been shown to activate MAP-2 kinase (ERK2) through stimulation of PKC (Leevers & Marshall, 1992), augments transactivation by Jun (Binetruy *et al.*, 1991). Indeed, Ha-*ras* stimulates phosphorylation of Jun at the sites which are targeted by MAP kinase (Binetruy *et al.*, 1991), while mutation of these sites abolishes the effect of Ras, indicating that phosphorylation is essential for stimulation (Smeal *et al.*, 1991). Activation of Jun by this mechanism is thought to play a major role in oncogene cooperativity between Ras and Jun.

It is important to stress the pivotal role played by MAP kinase in signal transduction (reviewed by Thomas, 1992). [This family of kinases is known by several names, including mitogen activated protein (MAP) kinases, microtubule-associated protein-2 (MAP-2) kinases, myelin basic protein (MBP) kinases and extracellular signal-regulated protein kinases (ERKs). Individual members of the family characterized to date include p42^{mapk/erk2}, p44^{mapk/erk1} and p54^{mapk}. In this review the MAP kinases will be referred to collectively as MAP kinase.] MAP kinase is activated by a number of external ligands [including nerve growth factor, epidermal growth factor, platelet-derived growth factor, fibroblast growth factor and insulin (Hoshi *et al.*, 1988; Ray & Sturgill, 1988; Rossomando *et al.*, 1989; Ahn *et al.*, 1990; Gomez *et al.*, 1990; Miyasaka *et al.*, 1990; Boulton *et al.*, 1991)] which mediate their signals through tyrosine kinase receptors. These molecules trigger phosphorylation of multiple cellular proteins on tyrosine residues and activate a protein kinase cascade which leads to rapid phosphorylation and activation of MAP kinase. MAP kinase itself is phosphorylated by MAP kinase kinase which targets both a threonine and a tyrosine residue in the protein (Anderson *et al.*, 1990; Gomez & Cohen, 1991). Earlier events in the signal transduction pathway (occurring between MAP kinase kinase and the receptors at the cell membrane) involve Ras and PKC, both of which lead to activation of MAP kinase activity (Rossomando *et al.*, 1989; Leevers & Marshall, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). However, a clear link between MAP kinase kinase and the early steps of the pathway has not yet been established. MAP kinase targets a range of substrates, including nuclear proteins such as Jun and Myc (both discussed in this Review). The involvement of MAP kinase in mediating the transmission of external signals to the nucleus is therefore central to the mechanism of nuclear signal transduction.

One additional interesting aspect of the *N*-terminal phosphorylation of Jun is the role of the delta domain which is located *N*-terminal to the MAP kinase phosphorylation sites (Fig. 3a). *In vivo*, v-Jun, which lacks the delta region, is underphosphorylated (at *N*-terminal phosphorylation sites) when compared with c-Jun and experiments using hybrid v-Jun/c-Jun proteins have correlated this reduction in phosphorylation with the absence of the delta domain (Black *et al.*, 1991). [It is also interesting that v-Jun is not a substrate for MAP kinase *in vitro* although Ser-62 and Ser-72 are present (D. Gillespie, personal communication).] Therefore, although the delta domain itself is not phosphorylated (Black *et al.*, 1991) it influences the phosphorylation state of Jun. The mechanism by which this occurs has not been determined. However, it is possible that removal of the delta domain allows Jun to adopt a constitutively active conformation resembling the phosphorylated, active c-Jun, and thereby removes the need for phosphorylation to switch on activity. Such a conformational change could block access of a kinase(s) to its target residue(s), explaining why v-Jun is underphosphorylated.

The *fos* gene was discovered as the oncogene of the FBR and FBJ murine osteosarcoma retroviruses (for reviews see Abate &

Curran, 1990; Woodgett, 1990; Karin, 1991a,b; Lewin, 1991). Fos does not bind to DNA itself, but its association with Jun cooperatively enhances the DNA binding activity of Jun to the TRE. In addition to its transactivation function, Fos is also a transcriptional repressor (Sassone-Corsi *et al.*, 1988). Repressor activity maps to regions of Fos (including the C-terminus) which are discrete from the transactivation domain and does not require interaction with the TRE (see Fig. 3b; Abate *et al.*, 1991a; Sassone-Corsi *et al.*, 1988; Lucibello *et al.*, 1988; Wilson & Treisman, 1988).

Initial interest in the potential role of phosphorylation in regulating Fos came from comparative analyses of c-Fos with the v-Fos transforming protein of the FBJ virus, one of three transforming retroviruses encoding activated Fos proteins. Both c-Fos and v-Fos from FBJ virus are phosphorylated at multiple common (as yet unidentified) sites *in vivo* (Barber & Verma, 1987). However, c-Fos is more extensively phosphorylated than v-Fos (by about 2–3-fold) and contains unique sites of phosphorylation which lie close to the C-terminus, within the 40-amino-acid region of c-Fos which is substituted by other sequences in the v-Fos protein (Barber & Verma, 1987). This region is also required for the transcriptional repression activity of c-Fos (Sassone-Corsi *et al.*, 1988; Abate *et al.*, 1991a). Stimulation of cells with TPA or serum increases the phosphorylation of c-Fos but not v-Fos and occurs only at this unique C-terminal segment (Barber & Verma, 1987). It does not, however, appear to involve PKC directly (Barber & Verma, 1987; Abate *et al.*, 1991b). These studies indicated that a region that has been lost during viral transduction is a target for regulation by hormonal signals and suggested that loss of phosphorylation within this region may lead to oncogenic activation of Fos, perhaps by regulating transrepressor function.

More recent studies have explored the effects of phosphorylation of c-Fos at its C-terminus. Substitution of either of three groups of serine residues within this domain by alanine residues [(i) Ser-362–364; (ii) Ser-368, -369 and -371; and (iii) Ser-373 and -374; see Fig. 3b] gives rise to Fos proteins which cannot repress transcription of the Fos promoter, but retain the ability to transactivate transcription from other promoters (Ofir *et al.*, 1990). In addition, mutation of residues 362–364 gives rise to a Fos protein which is activated for cellular transformation (Tratner *et al.*, 1992), supporting the theory that loss of this region in virally-transduced v-fos oncogenes contributes to oncogenicity. The finding that a Fos protein with negatively charged amino acids at positions 362–364 retains transrepression activity provides strong evidence that phosphorylation activates transrepression (Ofir *et al.*, 1990). Fos is therefore an excellent example of a nuclear protein with different biochemical activities which are regulated independently by phosphorylation.

Serines 362–364 lie within a consensus sequence for PKA, suggesting that this is one kinase which may phosphorylate Fos. Two groups have now confirmed that PKA phosphorylates Fos *in vitro* with high efficiency at Ser-362 (Abate *et al.*, 1991b; Tratner *et al.*, 1992). PKA is also likely to phosphorylate Fos *in vivo* since forskolin treatment of cells leads to increased phosphorylation of Fos (Tratner *et al.*, 1992). One biological consequence of this interaction is that since Fos represses its own transcription, the cAMP pathway can regulate the cellular level of Fos protein.

Fos is a substrate for several other protein kinases. PKC, cdk1 and an undefined nuclear kinase from HeLa cells are able to phosphorylate Fos *in vitro*; CKII, however, does not appear to phosphorylate Fos, even though a potential consensus CKII site is present (Abate *et al.*, 1991b). [cdk1 (cyclin-dependent protein kinase 1) is synonymous with p34^{cdc2}, the major cell division cycle-regulated protein kinase.] Although the target regions for

these kinases have been mapped (cdk1 also phosphorylates the C-terminal transrepression domain), the specific residues which are phosphorylated are not known, nor is it yet certain whether these kinases phosphorylate physiological sites or affect Fos activity (Abate *et al.*, 1991b). One particularly interesting observation is that Fos can be phosphorylated *in vitro* by double-stranded DNA-activated protein kinase (dsDNA-PK; Lees-Miller & Anderson, 1991). Indeed, Fos is the best substrate for dsDNA-PK so far identified (Lees-Miller & Anderson, 1991). This large serine/threonine kinase (reviewed by Lees-Miller and Anderson, 1991) has a molecular mass of 350 000, is potentially activated by low amounts of double stranded DNA (Lees-Miller *et al.*, 1990) and targets other nuclear proteins which are involved in transcription (Lees-Miller & Anderson, 1991), including T antigen and p53 (see below). The physiological significance of phosphorylation of Fos by this kinase is unknown.

In summary, regulation of the transcription factor AP-1 by different mitogenic signals can occur through phosphorylation of both the Jun and Fos proteins. Future studies should extend our knowledge of the enzymes which target these proteins physiologically and the effects of covalent modulation on the interaction of the AP-1 transcription factor with both the transcriptional machinery and its DNA recognition element.

NF- κ B

Nuclear factor kappa B (NF- κ B) was originally identified as a transcriptional activator which mediates its effects through binding to an enhancer element (κ B) within the κ light chain immunoglobulin gene (reviewed by Baeuerle & Baltimore, 1991; Karin, 1991a). NF- κ B is present in most cell types and can be activated in response to a variety of signals. The active form of the protein is a heterodimer comprising a 50 kDa DNA-binding subunit and a 65 kDa subunit. The p50 subunit is highly related to the proto-oncogene product Rel which also mediates its effects through the κ B enhancer.

NF- κ B is regulated at several levels including maturation by proteolysis, positive transcriptional autoregulation and phosphorylation. Of these, activation by phosphorylation in response to extracellular signals is particularly interesting because it occurs by a fascinating and unusual mechanism. In non-stimulated cells, NF- κ B is predominantly cytoplasmic and is maintained in an inactive form, which does not bind to DNA, through its association with a 37 kDa inhibitory protein called I κ B (Baeuerle & Baltimore, 1988). Phorbol esters, which activate PKC, promote dissociation *in vivo* of the NF- κ B-I κ B complex and migration of active NF- κ B into the nucleus (Sen & Baltimore, 1986). Moreover, studies *in vitro* have shown that PKC phosphorylates the I κ B component of the complex, leading to release of active NF- κ B which is able to bind to DNA (Shirakawa & Mizel, 1989; Ghosh & Baltimore, 1990). These findings form the basis for a model for NF- κ B activation, in which the protein is sequestered in the cytoplasm as an inactive complex with I κ B and released upon phosphorylation of the inhibitory subunit. The free NF- κ B heterodimer is then translocated into the nucleus where it binds to regulatory κ B motifs and activates transcription.

Other signals may be mediated through phosphorylation of I κ B. For example, haem-regulated eIF2 kinase (HRI) phosphorylates I κ B and promotes dissociation of the NF- κ B-I κ B complex (Ghosh & Baltimore, 1990). A similar mechanism may be involved in the activation of NF- κ B by double-stranded RNA, which activates a kinase closely related to HRI (Ghosh & Baltimore, 1990). NF- κ B is also activated by interleukin-1 through a cAMP-dependent mechanism (Shirakawa & Mizel, 1989). In this case the NF- κ B-I κ B complex is not a substrate for PKA, but phosphorylation of an as-yet-unidentified protein by

PKA is thought to mediate activation (Ghosh & Baltimore, 1990; Shirakawa & Mizel, 1989).

In conclusion, activation of NF- κ B involves an unusual mechanism in which protein phosphorylation of a cytoplasmic complex releases an active component which is then translocated to, and functions within, the nucleus.

Myc

The *myc* gene was originally discovered as a retrovirally transduced oncogene, *v-myc*, in the genomes of four isolates of highly oncogenic avian acute leukaemia retroviruses (reviewed in Luscher & Eisenman, 1990a; Penn *et al.*, 1990). The cellular counterpart, *c-myc*, was discovered subsequently. Genetic alteration of the *myc* locus is a common event in a large variety of both animal and human cancers and occurs by a number of mechanisms including pro-viral integrations, amplifications, point mutations and chromosomal translocations, all of which can lead to constitutive high level expression of the gene. For example, the human Burkitts lymphoma is characterized by reciprocal translocation between one of the immunoglobulin loci and the *c-myc* locus (Luscher & Eisenman, 1990a; Penn *et al.*, 1990).

c-Myc is now known to belong to a family of related proteins which also includes N-Myc and L-Myc. All of these are short-lived phosphoproteins which are located in the nucleus and bind DNA non-specifically (reviewed in Luscher & Eisenman, 1990a). Myc has recently been shown to bind specifically to the DNA sequence CACGTG (Blackwell *et al.*, 1990). A companion protein termed Max has recently been found in association with all three of these proteins *in vitro*, and with c-Myc and N-myc *in vivo*; Max association allows tighter binding to the motif CACGTG and is therefore thought to be important for Myc function (Blackwood & Eisenman, 1991; Wenzel *et al.*, 1991; Blackwood *et al.*, 1992). At the molecular level, Myc proteins are thought to behave as transcription factors, based on the following observations: (i) Myc shares structural similarity (based on sequence alignments) with other transcription factors; (ii) hybrid proteins comprising the N-terminal sequences of Myc and the DNA binding domain of the yeast GAL4 protein are potent transactivators of reporter constructs containing the GAL4 upstream activator sequence (Lech *et al.*, 1988; Kato *et al.*, 1990); (iii) Myc has sequence-specific DNA binding activity (Blackwell *et al.*, 1990) and (iv) substitution of the yeast PHO4 transcriptional activator protein with the DNA binding region of Myc (PHO 4 binds the same DNA sequence as Myc) leads to transactivation of the yeast acid phosphatase gene PHO5 via this sequence (Fisher *et al.*, 1991).

The biological role of Myc phosphorylation and the signalling pathways which target Myc have recently gained considerable interest. Myc is phosphorylated *in vitro* by at least three known protein kinases (Fig. 4). One of these, GSK3, phosphorylates Myc within the proline/glutamine-rich domain but little else is known about this interaction at present (Luscher & Eisenman, 1990a). CKII multiply phosphorylates c-Myc *in vitro* at two distinct regions, corresponding to amino acids 240–262 and 342–357 in the human c-Myc (Fig. 4; Luscher *et al.*, 1989). [CKII has also been shown to phosphorylate similar sites on the N-Myc protein (Hamann *et al.*, 1991)]. These sites are also phosphorylated *in vivo* (Luscher *et al.*, 1989) and lie, respectively, within an acidic central region (240–262), and a segment (342–357) flanking the basic DNA-binding domain (Fig. 4). Both of these regions are conserved between all Myc proteins and are functionally important for cellular transformation (reviewed in Penn *et al.*, 1990; Luscher & Eisenman, 1990a). Phosphorylation at residues within these domains may therefore regulate protein

Fig. 4. Phosphorylation of the Myc and Max proteins

The c-Myc protein and the Myc-associated protein, Max, complex with each other through 'leucine zipper' motifs and function as a complex *in vivo*. They are shown schematically with the positions of functional and structural domains indicated. Numbering refers to amino acid residues. Regions containing phosphorylation sites are expanded and the amino acid sequences given, together with the known enzymes which mediate the phosphorylation and dephosphorylation reactions.

function. Surprisingly, mutation of the Ser-347 and -348 phosphorylation sites, which map close to the DNA-binding region, does not affect the transforming activity of Myc when co-expressed with the Bcr-Abl tyrosine kinase oncogene in Rat-1 cells (Street *et al.*, 1990). Transforming activity would therefore appear to be independent of phosphorylation of Ser-347 and -348. The functional significance of phosphorylation at these sites is therefore still unresolved but may still be important for Myc function. The role of the CKII phosphorylation site within the acidic domain has not been established either, but may be important, since earlier studies had shown that deletion of this region from v-Myc led to a restriction of the types of cell which could be transformed (Ramsay *et al.*, 1980; Ramsay & Hayman, 1982). Interestingly, Max, the companion protein of Myc, is also phosphorylated by CKII (Wenzel *et al.*, 1991; Blackwood *et al.*, 1992), but in this case, phosphorylation abolishes the specific DNA-binding activity associated with the Max homodimeric complex but not the heterodimeric complex (Berberich & Cole, 1992).

MAP kinase [previously designated epidermal growth factor receptor threonine (ERT) kinase] has also been shown to phosphorylate Myc on Ser-62 both *in vitro* and *in vivo* (Alvarez *et al.*, 1991; Seth *et al.*, 1991; Pelech & Sanghera, 1992). No function has as yet been given to this site, but since it lies within a region which is required for transcriptional activation (Lech *et al.*, 1988; Kato *et al.*, 1990) and for the proposed association with the product of the retinoblastoma susceptibility gene (Rustgi *et al.*, 1991), its phosphorylation could regulate these associations.

Myb

Myb is a short-lived nuclear protein which is encoded by the proto-oncogene *c-myb* (for reviews, see Luscher & Eisenman, 1990b; Weston, 1990; Shen-Ong, 1990). Oncogenically active forms of the protein, encoded by *v-myb* genes of the avian myeloblastosis virus (AMV) and E26 transforming retroviruses, are truncated at both the N- and C-termini. Myb has recently been observed to be a transcription factor, both *in vitro* and *in vivo*, mediating its effects through interaction with a DNA sequence element (PyAACG/TG) termed the Myb response

element (MRE; Biedenkapp *et al.*, 1988). Several regions, including the DNA binding and transactivation domains, have been mapped within both the v- and c-Myb proteins (Fig. 5). In addition, c-Myb contains a C-terminal negative regulatory domain, deletion of which activates the transcriptional activation potential of the protein, and N-terminal sites which are modified by phosphorylation. Expression of Myb is restricted mainly to haematopoietic cells. Elevated expression of the protein can lead to stimulation of cell proliferation and inhibition of differentiation, suggesting that it may play a role in regulating the growth and differentiation of cells of haematopoietic lineage.

Myb is multiply phosphorylated *in vivo* at probably five sites (as defined by tryptic phosphopeptide mapping; Luscher *et al.*, 1990). Only two of these, Ser-11 and -12, have been identified to date (Luscher *et al.*, 1990). *In vitro*, Myb is an excellent substrate for CKII which targets both of these established *in vivo*

Fig. 5. Phosphorylation of the Myb protein

The 75 kDa product of the cellular *c-myb* gene is shown schematically with the DNA-binding domain and the CKII phosphorylation site highlighted. The phosphorylated residues, Ser-11 and -12, are shown (in bold) together with the flanking N- and C-terminal amino acids. Regions of the protein which are encoded by the transforming viruses E26 and AMV are indicated.

phosphorylation sites (Luscher *et al.*, 1990b). Although separated from the DNA-binding domain by more than 50 residues, phosphorylation at these two residues potentially down-regulates the ability of Myb to bind to its target sequence, the MRE, *in vitro* (Luscher *et al.*, 1990). This striking observation clearly demonstrated that phosphorylation by CKII, which targets many growth-related nuclear proteins, has a direct effect on the activity of a nuclear protein *in vitro*, and may regulate the function of this protein *in vivo*. It will therefore be interesting to see whether any signals modulate the phosphorylation of Ser-11 and -12 *in vivo*.

There is further evidence from oncogenic activation of Myb to support a role for Ser-11 and -12 in down-regulation of Myb activity *in vivo*. Firstly, the activated N-terminally truncated v-Myb proteins which are encoded by AMV and E26 lack the CKII phosphorylation sites (Weston & Bishop, 1989). Secondly, there are many examples of murine myeloid leukaemias and bursal lymphomas where precise retroviral integrations have occurred removing the initial exons containing these CKII sites (Luscher & Eisenman, 1990b, and references therein). These virally-activated Myb proteins are constitutively active with respect to MRE binding as, having lost three phosphorylation sites, they cannot be down-regulated by phosphorylation. In support of this idea, v-Myb or N-terminally truncated c-Myb proteins are no longer substrates for CKII *in vitro* and retain MRE DNA-binding activity under conditions in which MRE binding of wild-type c-Myb is blocked by CKII phosphorylation (Luscher *et al.*, 1990). Further analysis of these phosphorylation sites, perhaps by site-directed mutagenesis, should confirm whether it is loss of phosphorylation *per se*, or loss of other N-terminal residues, which is the basis of unregulated DNA binding activity.

Little else is known about Myb phosphorylation at present. There are clearly other sites which are phosphorylated *in vivo* (Luscher *et al.*, 1990). One possible contender for targeting these sites *in vivo* is glycogen synthase kinase 3 (GSK3) which has already been discussed as a nuclear kinase with respect to its phosphorylation of Jun (see above). Three closely spaced residues in c-Myb are phosphorylated by GSK3 *in vitro* and a major Myb kinase co-purifies with GSK3, suggesting that it might target Myb *in vivo* (Woodgett, 1991). Future studies should reveal the

functional significance of this interaction and the signals which control the phosphorylation of Myb.

THE LARGE T ANTIGEN OF SV40

The large tumour antigen (T antigen) of simian virus 40 (SV40) is a 708-amino-acid protein which is expressed early after SV40 infection. This fascinating molecule encompasses, within a single polypeptide, a number of biochemical activities which are required for the initiation of viral DNA synthesis and the co-ordination of events in viral replication (Livingston & Bradley, 1987). Viral propagation requires expression of the early region (including T antigen) which then permits replication of the viral genome and expression of the late region encoding the viral coat proteins. Replication activities include site-specific DNA binding, ATPase and DNA unwinding activities, all of which are essential for viral DNA replication. DNA binding occurs at two sites (termed sites I and II) at the viral origin of replication; binding to site I acts to repress transcription of the early region and augments viral DNA replication, whereas binding to site II is the first step in initiating viral DNA replication. In addition to transcriptional repression, T antigen is a transactivator of late transcription of viral genes. Complex formation of T antigen with two 'tumour suppressor' proteins, pRB (the product of the retinoblastoma susceptibility gene) and p53 (see below), is thought to stimulate the cell's replication machinery to accommodate SV40 replication, and is also thought to be (at least part of) the mechanism by which SV40 can cause cellular transformation.

T antigen is phosphorylated at multiple sites which are clustered into two clearly distinct domains (Kress *et al.*, 1982; Scheidtmann *et al.*, 1982, 1991a; Van Roy *et al.*, 1983; see Fig. 6). At the N-terminus, phosphorylation occurs at Ser-106, 111, 112, 120, 123 and Thr-124. Residues 106–112 lie within a domain which is conserved among papovaviruses (Moran, 1988) and is involved in cellular transformation and complex formation with the pRB protein (DeCaprio *et al.*, 1988). Ser-120 and -123 and Thr-124 lie adjacent to the nuclear localization signal (Kalderon *et al.*, 1984) and origin binding domain. The C-terminal phosphorylation sites, Ser-639, 676, 677 and 679 and Thr-701, lie

Fig. 6. Phosphorylation of the large T antigen of simian virus 40

T antigen, comprising 708 amino acids, is shown schematically. The symbols are as follows: NL, nuclear localization sequence; Rb, binding site for the retinoblastoma protein; ori binding, region implicated in specific binding to the SV40 origin of replication; Zn finger, zinc finger. Regions involved in cellular transformation, transcriptional transactivation, host range functions, ATPase activity, and interaction with the host proteins DNA polymerase α and p53 are indicated. Numbering refers to amino acid residues. Regions containing phosphorylation sites are expanded and the amino acid sequences given, together with the known enzymes which mediate the phosphorylation and dephosphorylation reactions.

close to or within a domain required for the host range functions of T antigen.

Several protein kinase activities which phosphorylate T antigen *in vitro* have been identified, suggesting that the protein is targeted by different signals. Both Ser-106 and Ser-112 are phosphorylated by CKII *in vitro* (Grasser *et al.*, 1988; Fig. 6) which again implicates this enzyme in regulating growth. Casein kinase I phosphorylates Ser-123 *in vitro* (Grasser *et al.*, 1988). Phosphorylation at this residue may depend on prior phosphorylation at Ser-120 to generate a recognition determinant (Flotow *et al.*, 1990; Scheidtmann *et al.*, 1991a; Umpruss *et al.*, 1992), but the kinase which phosphorylates Ser-120 is not known. Thr-124 is phosphorylated by the cell cycle-regulated kinase cdk1 (p34^{cdc2}) *in vitro* (McVey *et al.*, 1989) and Ser-677 has recently been shown to be phosphorylated *in vitro* by dsDNA-PK (Lees-Miller *et al.*, 1990; Chen *et al.*, 1991). Protein kinase activities which target other phosphorylation sites in T antigen have yet to be identified.

T antigen is an excellent example of a nuclear protein whose individual functions are regulated by separate phosphorylation events at different sites. The best-characterized effect of T antigen phosphorylation is its role in SV40 DNA replication (recently reviewed by Prives, 1990), where a 'push-pull' mechanism of stimulatory and inhibitory phosphorylation events controls the activity of the protein. Early studies showed that removal of serine phosphates from T antigen stimulates initiation of DNA synthesis *in vitro* by several-fold (Mohr *et al.*, 1987; Grasser *et al.*, 1987). This effect appears to be mediated solely by increased binding of T antigen to site II of the origin of replication (Simmons *et al.*, 1986; Mohr *et al.*, 1987; Klausning *et al.*, 1988). These studies suggested that phosphorylation of a serine residue(s) down-regulates origin-specific DNA binding and consequently the replication function of T antigen. Further evidence for control of replication by phosphorylation came from two different approaches. In the first, T antigen expressed in bacterial cells was shown to be inactive in replicating SV40 DNA *in vitro* (McVey *et al.*, 1989). However, when the bacterially expressed protein was phosphorylated at Thr-124 (by cdk1 *in vitro*), it acquired a hyperactive role in replication (i.e. more active than T antigen from HeLa cells, which is phosphorylated at this and at other sites) and bound extremely efficiently to site II of the replication origin (McVey *et al.*, 1989). These findings indicated a positive role for phosphorylation of T antigen at Thr-124 and suggested that phosphorylation at another site(s) down-regulates activity. Consistent with this idea, mutation of Thr-124 to alanine abolished the ability of T antigen to replicate SV40 DNA or produce viable virus particles, while mutation of Ser-679 enhanced replication by 5-fold and bound more tightly to site II than wild type T antigen (Schneider & Fanning, 1988). Ser-679 may therefore mediate (at least in part) the inhibitory effect on replication highlighted by the phosphatase studies. Other sites have also been implicated in controlling replication by the finding that protein phosphatase 2A (PP2A) catalytic subunit [which is equivalent to the replication factor RP-C (Virshup & Kelly, 1989)] specifically dephosphorylates Ser-120, 123, 677 and 679, and activates DNA replication (Scheidtmann *et al.*, 1991c; Virshup *et al.*, 1989; Lawson *et al.*, 1990). These studies also suggested a physiological role for PP2A in regulating T antigen-dependent replication. Moreover, G₁ cell extracts promote SV40 DNA replication less efficiently than do S phase extracts (Roberts & D'Urso, 1988), but addition of PP2A to the G₁ extract restores full activity (Virshup *et al.*, 1989). Based on these findings, it has been suggested that PP2A or a highly related phosphatase activity is regulated in a cell-cycle-dependent manner (Prives, 1990). Concomitant activation of cdk1 and PP2A at G₁/S could therefore switch T antigen into an active replicating protein in a

cell-cycle-dependent manner, thus allowing exploitation of the cell's replication machinery during S phase. It is also possible that the small t antigen of SV40, which down-regulates the activity of PP2A towards T antigen at the inhibitory phosphorylation sites (Yang *et al.*, 1991; Scheidtmann *et al.*, 1991b), is necessary to maintain T antigen in an inactive replication state until its activation at an appropriate point in the cycle. Prior to S phase, T antigen may be employed in other function(s) such as transcription or tumour suppressor protein binding.

In addition to its effects on DNA replication, phosphorylation of T antigen may have profound effects on other biological and biochemical activities associated with this protein. For example, mutation of Ser-677 abolishes binding to site I in the origin (Schneider & Fanning, 1988), and could therefore be involved in regulating repression of early SV40 transcription. However, although mutation of this site reduces viability of the virus slightly, there is not yet any direct evidence for a role in repression. Mutagenesis experiments have also shown that three residues are important for the transforming ability of T antigen. Loss of phosphorylation at Ser-639 causes a stimulation of cellular transformation (Schneider & Fanning, 1988; Scheidtmann *et al.*, 1991a), but the mechanism of this effect is not yet known. Phosphorylation of Ser-679 and especially Ser-677 are also thought to be important for transformation but in contrast to the loss of Ser-639, mutation of either of these sites gives rise to T antigen molecules which do not transform cells (Schneider & Fanning, 1988; Scheidtmann *et al.*, 1991a). Once again, both stimulatory and inhibitory phosphorylation events appear to control a function of T antigen. This result also suggests a role in transformation for dsDNA-PK which phosphorylates this site *in vitro* (Chen *et al.*, 1991). The mechanism by which phosphorylation at this site influences transformation is not clear. However, phosphorylation of a mutant T antigen lacking Ser-677 is reduced at Ser-120 and -123 and enhanced at Ser-112 (Scheidtmann *et al.*, 1991a), suggesting that modification of Ser-677 may influence the phosphorylation state of other regulatory sites. Further evidence for the involvement of these sites in transformation has come from a fascinating study describing Rev2, a cell line originally transformed by SV40, which has a revertant (non-transformed) phenotype (Deppert *et al.*, 1991). The T antigen expressed in this line is wild type and when rescued, retains the ability to fully transform other cell lines. Yet in the Rev2 background, expression of wild type T antigen is not transforming. Analysis of T antigen in Rev2 cells showed significant loss of phosphorylation at Ser-120, 123, 667 and 679 (Deppert *et al.*, 1991) suggesting that phosphorylation at these sites may favour a transformation competent conformation of T antigen. The nature of the reversion is at present unknown, but it may inactivate a kinase(s) required for T antigen phosphorylation. It will be interesting to determine whether dsDNA-PK is active in these cells and whether expression of this kinase can restore transformation competence.

Another potentially interesting role for phosphorylation is in the transport of T antigen to the nucleus. Although nuclear localization is dependent on a specific sequence of amino acids (residues 126–132; Kalderon *et al.*, 1984), the rate of transport is influenced by residues 111–125 (Rihs & Peters, 1989) which contains several phosphorylation sites. Subsequent work has identified the casein kinase II site (Ser-112) as the determining factor for rapid nuclear transport (Rihs *et al.*, 1991) and defined a biological role for phosphorylation of this residue.

Finally, although considerable progress has been made in understanding how the replication and transformation functions of T antigen are controlled, there are still many questions to be answered. The phosphorylation requirements for other activities

such as transcriptional activation, repression and binding to the pRB and p53 suppressor proteins are still unknown and it is therefore possible that phosphorylation at specific sites plays a major role in controlling individual functions.

pRB

The retinoblastoma susceptibility gene (*RB*) was originally identified as the genetic locus for predisposition to retinoblastoma (reviewed in Weinberg, 1990). Mutation or deletion of both alleles of *RB* occurs in most retinoblastomas and in several other types of human cancer, indicating that loss of *RB* function is a major event in the pathogenesis of many forms of this disease (Lee *et al.*, 1988). Reintroduction of the *RB* gene into cells which lack a functional *RB* has been shown to suppress several neoplastic phenotypes and abolish tumorigenic growth in nude mice (Huang *et al.*, 1988; Bookstein *et al.*, 1990). *RB* has therefore been classed as a tumour suppressor gene, the normal function of which is to negatively regulate cellular proliferation. The product of the *RB* gene (pRB) is a nuclear protein of molecular mass 110000 (Friend *et al.*, 1986; Lee *et al.*, 1987a,b; Fung *et al.*, 1987) which is multiply phosphorylated (Ludlow *et al.*, 1989; Buchkovich *et al.*, 1989). The transforming proteins of several DNA tumour viruses interact with pRB and are thought to block its suppressor function, thereby contributing to the mechanism of transformation by these viruses (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989).

There are two striking aspects of pRB phosphorylation. The

first is that the SV40 large T antigen complexes exclusively with unphosphorylated pRB (Ludlow *et al.*, 1989) suggesting that unphosphorylated pRB is the active suppressor. The second is that pRB undergoes striking cell-cycle-dependent changes in its phosphorylation state (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989; Xu *et al.*, 1989; Ludlow *et al.*, 1990; Furukawa *et al.*, 1990a), being predominantly unphosphorylated during the G₁ phase of the cell cycle [where control of cell proliferation often occurs (Pardee, 1989)], but hyperphosphorylated throughout S and G₂/M (Fig. 7). The occurrence of a major phosphorylation event at the G₁/S boundary suggests that phosphorylation of pRB might signal commitment to continuation of the cell cycle either by promoting the onset of S phase or determining whether post-mitotic cells enter the G₀ (quiescent) state or re-enter G₁ (Cooper & Whyte, 1989). This idea is consistent with the conclusion that T antigen targets only the active (unphosphorylated) species of pRB which is present at G₁ and explains the ability of viral oncoproteins to stimulate cellular DNA synthesis and block differentiation.

There are several lines of evidence which support the idea that unphosphorylated pRB actively arrests cell growth. Firstly, pRB is rapidly dephosphorylated by agents which promote cellular differentiation or cell cycle arrest (Akiyama & Toyoshima, 1990; Chen *et al.*, 1989; Mihara *et al.*, 1989), including physiological growth inhibitors such as α -interferon (α -IFN) (Thomas *et al.*, 1991) and transforming growth factor β 1 (TGF β 1) (Laiho *et al.*, 1990). Interestingly, TGF β 1 does not arrest cells which express T antigen, presumably because T antigen binds unphosphorylated pRB. However, in these cells, TGF β 1 retains the ability to block pRB phosphorylation (Laiho *et al.*, 1990). This observation indicates that inhibition of pRB phosphorylation is not a consequence of growth arrest, but favours the idea that dephosphorylation of pRB is a key step in the mechanism which brings about growth arrest. A second line of evidence is that terminally differentiated or senescent cells cannot enter S phase following mitogenic stimulation and are unable to phosphorylate pRB (Furukawa *et al.*, 1990a; Stein *et al.*, 1990), even although they undergo the same prereplicative events as stimulated quiescent cells (Stein *et al.*, 1990). This suggests that phosphorylation of pRB is required to re-enter the cell cycle. Finally, microinjection of unphosphorylated pRB directly arrests cell cycle progression in mid-G₁ phase; this block can be alleviated by T antigen (Goodrich *et al.*, 1991). Taken together, these findings suggest that dephosphorylation of pRB is an integral part of the mechanism of growth arrest.

The functional consequences of pRB phosphorylation at the molecular level are still unclear, but several recent studies have begun to address this question. At least seven cellular proteins can complex with pRB *in vitro* (Kaelin *et al.*, 1991; Huang *et al.*, 1991), including the transcription factors c-Myc (Rustgi *et al.*, 1991) and E2F (Bandara & La Thangue, 1991; Chellappan *et al.*, 1991; Bagchi *et al.*, 1991; Chittenden *et al.*, 1991). pRB is therefore thought to act as a transcriptional repressor by sequestering key proteins which are required for cell cycle progression (Wagner & Green, 1991). Complex formation with E2F occurs only with unphosphorylated pRB and is restricted to G₁ (Chellappan *et al.*, 1991). Others have shown that unphosphorylated pRB is tightly associated with subnuclear structures and is released from these complexes in late G₁ or early S, coincident with the major pRB phosphorylation event (Mittnacht & Weinberg, 1991). Based on these studies, it is likely that phosphorylation of pRB at a specific control point in G₁ (or complex formation with oncoproteins) dissociates these subnuclear structures and, by releasing the sequestered proteins, allows the cell cycle to continue.

Fig. 7. Cell cycle-dependent phosphorylation of the retinoblastoma tumour suppressor protein, pRB

Actively cycling cells contain predominantly phosphorylated pRB whereas non-cycling (or G₀) cells contain only the unphosphorylated form of the protein. During the cell cycle pRB becomes progressively phosphorylated throughout G₁ and culminates in a major phosphorylation event at the G₁/S boundary with the predominant appearance of the most highly phosphorylated form of the protein. Dephosphorylation is thought to occur as the cells pass from G₂/M to G₁ or G₀. Phosphorylation, or complex formation with oncoproteins such as the SV40 large T antigen (which associates only with the unphosphorylated pRB), is thought to inactivate the growth suppressor function of pRB.

The amino acid residues which are phosphorylated in pRB *in vivo* have not yet been rigorously identified, although peptide mapping has revealed that the protein contains more than 20 tryptic phosphopeptides (Lees *et al.*, 1991). A large subset of these phosphopeptides are also present in pRB which has been phosphorylated *in vitro* by cdk1 (p34^{cdc2}), suggesting that this kinase is able to phosphorylate the physiological targets within the pRB sequence (Taya *et al.*, 1989; Lin *et al.*, 1991; Lees *et al.*, 1991). [Several consensus sequences for recognition by this kinase are present in pRB, and synthetic peptides corresponding to these sequences have been used to identify some of the sites phosphorylated *in vitro* (Taya *et al.*, 1989; Lees *et al.*, 1991).] Immunodepletion of cdk1 kinase from cell extracts (Lin *et al.*, 1991) or expression of anti-cdk1 oligonucleotides (Furukawa *et al.*, 1990b) depletes the kinase activity towards pRB, suggesting that cdk1 or a highly related kinase phosphorylates pRB *in vivo*.

In addition, other protein kinases are also likely to be active towards pRB *in vivo*. For example, partially phosphorylated species of pRB have been observed in early G₁ prior to the major phosphorylation event at G₁/S (Chen *et al.*, 1989; Thomas *et al.*, 1990; Fukurawa *et al.*, 1990b). Moreover, TGF β 1 can only partially block pRB phosphorylation, suggesting that there may be at least two pRB kinases, only one of which is responsive to TGF β 1 (Laiho *et al.*, 1990). The identities of other kinase(s) which phosphorylate pRB are still unknown. Future studies should reveal the nature of these as well as clarify the biological roles of the differentially phosphorylated forms of pRB.

p53

The p53 tumour suppressor protein (Lane & Benchimol, 1990; Levine *et al.*, 1991) is a fascinating nuclear phosphoprotein which can potently suppress cellular transformation (Finlay *et al.*, 1989; Mercer *et al.*, 1990; Eliyahu *et al.*, 1989; Michalovitz *et al.*, 1990), inhibit tumorigenesis (Chen *et al.*, 1990), arrest cell growth (Baker *et al.*, 1990; Diller *et al.*, 1990) and induce apoptotic cell death (Yonish-Rouach *et al.*, 1991). Loss of the

ability of p53 to tightly regulate cell growth, through mutation, is the most common genetic alteration in most inherited and sporadic forms of human cancer (Levine *et al.*, 1991). The suppressor activity of p53 is also blocked by the products of viral oncogenes such as the SV40 large T antigen, the adenovirus E1B 55K protein and the E6 protein of human papilloma virus (HPV) types 16 and 18 (Fukasawa *et al.*, 1991; Scheffner *et al.*, 1990). The mechanism by which p53 regulates cell growth at the molecular level is still unknown. However, its ability to block SV40 DNA replication (Braithwaite *et al.*, 1987; Wang *et al.*, 1989; Sturtzbecher *et al.*, 1988) and interact with proteins involved in DNA replication (Gannon & Lane, 1987; Wilcock & Lane, 1991) have suggested that it might normally regulate the initiation of cellular DNA replication. Other studies have shown that p53 is a potent transcriptional activator (Raycroft *et al.*, 1990; Fields & Jang, 1990; O'Rourke *et al.*, 1990). p53 may therefore have a dual role in regulating cell growth. Understanding its mechanism of action and regulation is a major goal in cancer biology.

p53 is multiply phosphorylated by several different protein kinases (Fig. 8) and might therefore be controlled, perhaps coordinately, by more than one signal. Phosphorylation of p53 is also modulated by the transforming proteins of DNA tumour viruses, which suggests that it may play a central role in controlling the growth of the cell. There are two C-terminal phosphorylation sites in p53, Ser-309 and -386 (in mouse p53) and a cluster of phosphorylation sites at the N-terminus, including Ser-4, 6, 15 and 34 (Samad *et al.*, 1986; Meek & Eckhart, 1988). Different cell lines or species show similar but not identical phosphorylation patterns and additional, as yet unidentified, sites of phosphorylation may be present.

There has been considerable progress in identifying the protein kinases which phosphorylate p53. Ser-315 in human p53 (equivalent to Ser-309 in mouse p53) is phosphorylated *in vitro* by the cdk1 (p34^{cdc2}) kinase (Bischoff *et al.*, 1990; Addison *et al.*, 1990), suggesting that cell-cycle-dependent phosphorylation might control p53, perhaps as a fine tuning mechanism, in addition to the cell-cycle-regulated expression of p53 (Reich &

Fig. 8. Phosphorylation of the p53 tumour suppressor protein

The mouse p53 protein comprising 387 amino acids is shown schematically. The positions of functional and structural domains are indicated together with the positions of five highly conserved boxes which are numbered in Roman numerals. Other numbering refers to amino acid residues. Regions containing phosphorylation sites are expanded and the amino acid sequences given, together with the enzymes which mediate the phosphorylation and dephosphorylation reactions. The proposed position of attachment of RNA is indicated.

Levine, 1984). Evidence that p53 becomes more highly phosphorylated upon entering the S phase of the cell cycle supports this idea (Bischoff *et al.*, 1990). Moreover, since immunodepletion of cdk1 from cell lysates removes the kinase activity which phosphorylates Ser-315 (Bischoff *et al.*, 1990), cdk1 or a highly related kinase may well phosphorylate p53 *in vivo*. Whether this phosphorylation event regulates p53 function has not been established. Mutation of p53 at the cdc2 phosphorylation site in mouse p53 (Ser-309) has no effect on the ability of p53 to block replication of SV40 DNA in COS cells (Meek & Eckhart, 1990). However, some other activity such as growth suppression, transcriptional activation or perhaps nuclear localization (Addison *et al.*, 1990; Dang & Lee, 1989) may be regulated by this phosphorylation event. The finding that transformation by SV40 increases phosphorylation at this site would suggest that it does play an important role (Meek & Eckhart, 1988).

A second growth-related kinase, CKII, phosphorylates p53 *in vitro* at Ser-386 (Meek *et al.*, 1990; Herrmann *et al.*, 1991). Two lines of evidence, the association of p53 with CKII (or a CKII-like kinase) in p53 immunoprecipitates, and the co-purification of CKII with a p53 kinase from SV3T3 cells, support the idea that p53 is phosphorylated by CKII *in vivo* (Meek *et al.*, 1990; Herrmann *et al.*, 1991). Ser-386 is also the proposed site of attachment of an unusual RNA moiety (Samad & Carroll, 1991), but the nature of this RNA or its physiological significance are far from clear at present. Recent evidence suggests that phosphorylation at Ser-386 is required for activating p53 suppressor function since replacement of the serine with an alanine residue abolishes the ability of p53 to suppress growth (Milne *et al.*, 1992b). Placing a constitutive negative charge at this site (aspartate) partially restores suppressor activity, suggesting that it is able to mimic phosphorylation (Milne *et al.*, 1992b). It is not yet known whether any physiological signals or conditions of growth are able to modulate the phosphorylation of p53 at this site and hence regulate its effects on cell growth.

Two kinases phosphorylate p53 at N-terminal sites. One of these is the dsDNA-PK which phosphorylates an as-yet-unidentified serine residue(s) (Lees-Miller *et al.*, 1990). It is not yet known how this phosphorylation event may control p53 activity. Interestingly, p53 has been reported to be induced by DNA damage (Maltzman & Czyzyk, 1984); activation of dsDNA-PK by changes in the DNA structure (Lees-Miller & Anderson, 1991) could therefore mediate this signal. Such a mechanism could play a major role in the response of p53 to DNA damage preceding neoplastic transformation. Casein kinase I has also been shown to phosphorylate p53 at three N-terminal sites (Ser-4, 6 and 9; Milne *et al.*, 1992a). This is a monomeric serine/threonine kinase of molecular mass 37000, which is ubiquitous in eukaryotic cells and exists in both cytoplasmic and nuclear forms. A similar kinase, which is preferentially active towards p53, is present in SV3T3 cell lysates and has all the hallmarks of a CKI-related protein [four isoforms of casein kinase I have recently been identified by molecular cloning (Rowles *et al.*, 1991)]. Interestingly, a DNA repair-defective mutation (hrr25-1) in *Saccharomyces cerevisiae* lies within a gene encoding a protein kinase with striking similarity to CKI (Hoekstra *et al.*, 1991). Here again is a potentially exciting link between DNA damage, p53 and a protein kinase.

The effect of DNA tumour viral products on the phosphorylation state of p53 may provide considerable insight into understanding the regulation of p53 and its effect on growth. For example, transformation by SV40 has been reported to stimulate phosphorylation of p53, with overall increases generally in the range of 50–60% (Sturzbecher *et al.*, 1987). These changes may reflect increased phosphorylation only at a specific site or

sites (Samad *et al.*, 1986; Meek & Eckhart, 1988). In this respect, a recent study by Scheidtmann's group is particularly interesting. Analysis of SV40-transformed and untransformed rat cell lines revealed the presence of a novel, as yet unidentified, phosphorylation site present only in the transformed cells (Scheidtmann & Haber, 1990). This novel phosphorylation, which resulted in the stoichiometric shift of p53 to a faster-migrating form, was dependent on protein synthesis and suggested that T antigen may induce a cellular kinase(s). Similar observations have been made by others (Sturzbecher *et al.*, 1987). Of additional interest is the finding that the SV40 small t antigen may promote phosphorylation of p53 by blocking protein phosphatase 2A-dependent dephosphorylation of p53 (Scheidtmann *et al.*, 1991b). Taken together, these findings suggest that certain phosphorylation states of p53 may be important for efficient cellular transformation or viral replication. The nature and function of these phosphorylation states have still to be resolved.

CONCLUSIONS

In this Review, we have discussed a selected number of nuclear proteins which play a role in growth control and are regulated by reversible protein phosphorylation. Taken together, several common themes emerge from these examples.

(1) Many nuclear proteins are regulated by phosphorylation and the effect can be either stimulatory or inhibitory. Phosphorylation controls many different activities of nuclear proteins, including DNA binding, transactivation, protein-protein contact, subcellular localization and protein stability.

(2) Many proteins are phosphorylated at multiple sites by different protein kinases. Different signal transducing pathways can therefore converge on a particular protein, with the result that two signals could regulate activity co-ordinately or may have opposing effects. Similarly, in some cases a single residue within the protein is targeted by more than one protein kinase, indicating that different signals can share a regulatory mechanism.

(3) Different phosphorylation events can independently regulate different activities within a pleiotropic protein.

(4) Many signal-transducing protein kinases (or phosphatases) have broad substrate specificities, suggesting that they may be important for co-ordinate control of nuclear events. In particular, casein kinase II may well have a central role in regulating growth (Meisner & Czech, 1991).

(5) In some cases, a clearly established signal exists between the cell membrane or the cytoplasm and the modulation of protein activity in the nucleus.

(6) In the case of oncogene (or anti-oncogene) products, there is often a correlation between transforming ability and phosphorylation status of the protein. Moreover, DNA tumour viruses have evolved mechanisms that interrupt the regulatory circuitry in the nucleus, underlining the importance of phosphorylation in the regulation of growth control.

Finally, although the past few years have seen major advances in our understanding of the control of nuclear events by phosphorylation, many more questions remain than have been answered. Newly discovered or yet-to-be-discovered proteins may well be regulated by phosphorylation. In many cases, the functional relevance of phosphorylation of known proteins remains unclear, the enzymes that modify these proteins are unknown or uncharacterized, and the signals to which they respond are as yet undiscovered or poorly understood. Clearly there is much to be learned about protein phosphorylation in the nucleus and there is considerable scope for future study in what is an exciting and rapidly expanding area of biological research.

Answers to these questions will undoubtedly provide a better understanding about key areas of growth control such as differentiation, development and cellular transformation.

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