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The Human DNA-Activated Protein Kinase, DNA-PK: Substrate
Specificity.

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NOTE TO EDITOR: In the text and references, Greek letters are
spelled out and enclosed in square brackets, i.e. [beta].

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Introduction Agents that cause damage to DNA (DNA damage-inducing (DDI) agents) arrest cell cycle progression in all eukaryotes from yeast to humans at positions in late G_1 and G_2 that have become known as "checkpoints" (Hartwell and Weinert, 1989; Murray, 1992; Sheldrick and Carr, 1993; Weinert and Lydall, 1993), presumably to allow time for DNA repair. Otherwise the DNA damage would become irreversibly fixed as a consequence of DNA replication in S phase, or through cell division at mitosis (M phase). The mammalian G_2 checkpoint mechanism is not yet well characterized (O'Connor and Kohn, 1992), but the key observation that tumor cells with mutant p53 were unable to arrest in G_1 (Kastan et al., 1991) quickly led to an outline of the mammalian G_1 checkpoint mechanism (Hunter, 1993; Appella and Anderson, 1994; Appella et al., this volume). The p53 tumor suppressor gene is a transcription factor that normally is relatively inactive because it is rapidly degraded (Levine, 1993). However, in response to exposure to ultraviolet light, ionizing radiation, and other DDI agents, the p53 protein is transiently stabilized, accumulates in the cell nucleus, and induces the expression of several genes including WAF1 and GADD45 (El-Deiry et al., 1993; El-Deiry et al., 1994). The 21 kDa product of WAF1 is a potent inhibitor of the cyclin-dependent protein kinases that are needed for the transition from G_1 to S phase and for continued DNA replication in S (Dulić et al., 1994; Harper et al., 1993). Although the G_1 checkpoint mechanism probably is much more complex, the induction of WAF1 provides a simple explanation of how cell cycle progression can be arrested. In addition to WAF1, about 50 other genes are known to be induced in mammalian cells

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after exposure to DDI agents (Fornace, 1992; Herrlich and Rahmsdorf, 1994). Recent studies indicate that some genes are induced as a consequence of the effects of DDI agents on other cellular molecules and not necessarily as a consequence of damage to DNA (Anderson, 1994; Herrlich, and Rahmsdorf, 1994; Sachsenmaier et al., 1994). Such exposures activate cytoplasmic signaling mechanisms that operate through protein kinase cascades initiated at or near the plasma membrane; in turn, these kinase cascades activate several transcription factors including AP1 and NF-[kappa]B. Nevertheless, there is strong evidence that the p53-dependent induction of WAF1 is a direct consequence of the production of DNA strand breaks (Nelson and Kastan, 1994) and that DNA strand breaks are the signals for activation of the G₁ checkpoint(s) in yeast (Siede et al., 1994).

Although much has been learned about the structure and function of p53 and the probable sequence of subsequent events that lead to cell cycle arrest, little is known about how DNA damage is detected and the nature of the signal that is generated by DNA damage. Circumstantial evidence suggests that protein kinases may be involved. Indeed, several yeast kinase genes were identified by screening for mutants defective in their ability to arrest cell cycle progression after exposure to DDI agents (Walworth et al., 1993; Weinert et al., 1994; Allen et al., 1994; Anderson, 1994). In mammalian cells, the situation is less clear; however, in hamster cells, 2-aminopurine overrides the G₁, S, and G₂ checkpoints, and 2-aminopurine and H7, another protein kinase inhibitor, block DDI-agent induction of the GADD45 gene

(Andreassen and Margolis, 1992; Luethy and Holbrook, 1994).

Two moderately abundant nuclear enzymes have been described in mammalian cells that recognize DNA strand-breaks and transmit signals to other proteins. One is poly(ADP-ribose) polymerase (de Murcia and de Murcia, 1994; Satoh and Lindahl, 1992); this enzyme is activated by binding to nicks, and it ribosylates histones, other chromosomal proteins, and itself. It may be responsible for altering chromatin structure near sites of DNA damage and also may signal the presence of damage through transient changes in NAD levels. A second DNA structure-signaling enzyme is DNA-PK (Anderson, 1993). DNA-PK is activated by binding to DNAs with nicks, gaps, or double-strand breaks, and it may function, at least in part, as a detector of DNA strand-breaks.

The structure of DNA-PK. DNA-PK is believed to consist of a very large polypeptide, DNA-PK_C (or Prkdc for protein kinase, DNA-activated, catalytic component), that probably contains the catalytic site, and a DNA binding/targeting and regulatory subunit, which can be the Ku autoantigen (Dvir et al., 1992; Gottlieb and Jackson, 1993; Anderson, 1993). The large DNA-PK_C polypeptide and DNA-activated kinase activity co-purify (Lees-Miller et al., 1990; Carter et al., 1990). The size of the DNA-PK_C polypeptide was initially estimated from SDS-polyacrylamide gels electrophoresis to be 300-350 kDa; however, the size of the nascent polypeptide, estimated from preliminary sequence analysis of the ~13 kbp cDNA, is close to 450 kDa. The difference is

attributable to the lack of good molecular weight markers in this size range. Although nearly ten times the size of many protein kinase catalytic subunits, several findings are consistent with an assignment of the catalytic site to the 450 kDa DNA-PK_C polypeptide. DNA-PK_C binds ATP and can be labeled by the ATP analogues fluorosulfonylbenzoyladenine (FSBA) and azido-ATP; FSBA inhibits DNA-PK kinase activity (Lees-Miller et al., 1990). A monoclonal antibody specific for the DNA-PK_C polypeptide depleted DNA-dependent kinase activity from HeLa extracts (Carter et al., 1990). Finally, sequence analysis of the cDNA has revealed a segment with homology to other kinase catalytic domains. In the human cell lines that have been examined, DNA-PK_C is moderately abundant and predominantly nuclear (Anderson and Lees-Miller, 1992). We estimated its abundance to be about 50,000 molecules per cell, but our estimate involved several assumptions and an accurate measurement has not been made. DNA-PK activity is approximately 100-fold more abundant in extracts of human and monkey cell lines compared to extracts of rodent and insect cell lines; thus, DNA-PK activity and the DNA-PK_C polypeptide are difficult to detect with current assays in unfractionated extracts of non-primate cells (Anderson and Lees-Miller, 1992).

Ku was first recognized as a heterodimeric (p70/p80), nuclear, phosphoprotein that reacted with sera from patients suffering from the autoimmune diseases lupus erythematosus and scleroderma polymyositis (Mimori et al., 1981; Reeves, 1992). HeLa cells contain ~400,000 molecules of Ku per cell (Mimori et

al., 1986), but Ku also appears to be less abundant in non-primate cells and was not detected in mouse L-929 cells using mouse-specific monoclonal antibodies (Wang et al., 1993). In vitro Ku binds initially to the ends of linear DNA fragments but then can translate along the DNA in an ATP-independent manner (de Vries et al., 1989). Ku also recognizes DNAs with nicks and gaps, as well as DNAs with single- to double-strand transitions (Blier et al., 1993; Falzon et al., 1993), and these structures activate DNA-PK (Morozov et al., 1994). cDNAs for the two Ku polypeptides have been cloned and sequenced from both human and mouse cells (Chan et al., 1988; Reeves and Sthoeger, 1989; Yaneva et al., 1989; Mimori et al., 1990; Griffith et al., 1992; Porges et al., 1990; Falzon and Kuff, 1992), and the human genes recently were mapped to chromosomes 2 (p80) and 22 (p70) (Cai et al., 1994). The location of the p80 Ku subunit gene corresponds to the location of the human gene (XRCC5) that complements ionizing-radiation sensitivity in group 5 hamster cells. Transfection of the cDNA for the human Ku p80 subunit into group 5 hamster cells that are defective in repairing double strand breaks restored their X-ray sensitivity to normal levels and corrected the defect in site-specific recombination (Rathmell and Chu, 1994a,b; Getts and Stamato, 1994; Taccioli et al., 1994). Thus, Ku is likely to play a role in these processes. It may protect DNA ends from exonucleolytic degradation; however, by activating DNA-PK, it also might have other signaling functions. Recently, Ku was shown to have DNA helicase activity and to be identical to a previously described activity, human DNA helicase II (Tuteja et al., 1994); thus, Ku probably also performs

functions that are independent of DNA-PK_C. It is not known if other targeting proteins can substitute for Ku to activate DNA-PK_C, perhaps in response to other signals.

DNA-PK Substrate Specificity. In vitro, human DNA-PK phosphorylates a variety of nuclear DNA-binding, regulatory proteins including the tumor suppressor protein p53, the single-stranded DNA binding protein RPA, the heat shock protein hsp90, the large tumor antigen (TA_g) of simian virus 40, a variety of transcription factors including Fos, Jun, serum response factor (SRF), Myc, Sp1, Oct-1, TFIID, E2F, the estrogen receptor, and the large subunit of RNA polymerase II (reviewed in Anderson, 1993; Jackson et al., 1993). However, for most of these proteins, the sites that are phosphorylated by DNA-PK are not known.

To determine if the sites that were phosphorylated in vitro also were phosphorylated in vivo and if DNA-PK recognized a preferred protein sequence, we identified the sites phosphorylated by DNA-PK in several substrates by direct protein sequence analysis. Table 1 shows an alignment of known DNA-PK phosphorylation sites. Each phosphorylated serine or threonine is followed immediately by glutamine in the polypeptide chain; at no other positions are the amino acid residues obviously constrained.

Two forms of hsp90, designated [alpha] and [beta], are found in human cells, and these proteins are 97% identical; however, only hsp90[alpha] is phosphorylated by DNA-PK (Lees-Miller and

Anderson, 1989b). The phosphorylated sites are two threonines at the amino terminus of hsp90[alpha] in the sequence PEETQTQDQPM¹¹; these residues are not present in hsp90[beta]. SV40 TAg was shown to be phosphorylated at four sites, serines 120, 665, 667, and 677 (Chen et al., 1991). Isolation of the phosphopeptides containing these residues was accomplished using iron-affinity chromatography in conjunction with conventional reverse phase HPLC. The phosphorylated serines then were identified by direct sequence analysis after converting the phosphoserine to S-ethylcysteine. Each phosphorylated TAg serine is followed immediately by glutamine (Table 1). Serines 120 and 677 are phosphorylated in vivo, but serines 665 and 667 have not been shown to be in vivo sites of phosphorylation. TAg serine 639 is phosphorylated in vivo, and although this serine is followed by glutamine in the TAg polypeptide, it was not phosphorylated by DNA-PK in vitro. This finding suggests that either Ser639 is phosphorylated by a different kinase or that its phosphorylation by DNA-PK requires a particular TAg conformation that was not present in the in vitro reaction.

Subsequent to our initial work, Bannister et al. (1993) used a genetic approach to identify serine 249 in the DNA-binding region of Jun as the likely site of phosphorylation by DNA-PK. This site is phosphorylated in vivo, but it also can be phosphorylated in vitro by casein kinase II. Jun residue 250 is glutamine; changing it to alanine largely prevented DNA-dependent Jun phosphorylation, again suggesting that glutamine is important for substrate recognition by DNA-PK. Changing glutamic acid 251

to alanine decreased the rate of Jun phosphorylation about twofold, indicating that the residue in this position also may contribute to substrate recognition. Glutamic acid also is present at the +2 position with respect to the serine 15 site of human and mouse p53, and aspartic acid (D) is present at +2 after the second TQ site in hsp90 (Table 1, see below), but neither glutamic or aspartic acid are at this position in sites from SV40 Tag, or the human serum response factor (SRF). Two serines followed by glutamine in a peptide derived from the carboxy-terminal transactivation domain of SRF are phosphorylated by DNA-PK, and they also appear to be phosphorylated in serum-stimulated cells (Liu et al., 1993).

To examine further the role of glutamine in site recognition, we examined the ability of DNA-PK to phosphorylate synthetic peptides corresponding to segments of the human p53 protein sequence. These peptides covered all of the known phosphorylation sites in human p53, including serines 9, 15, 33, 315, and 392, and all -SQ- or -QS- sites (i.e. S15, -LSQE-; S37, -PSQA-, S99, -PSQK-, S166, QSQH, and S376, GQSTS) (Lees-Miller et al., 1992). Peptides containing three of the five SQ or QS motifs were phosphorylated by DNA-PK; one of these, serine 15, is in a highly conserved region and is phosphorylated in vivo (Ullrich et al., 1993). The sequence requirements for phosphorylation at this site were examined in more detail using a series of synthetic peptides (Table 2). Shortening the sequence to less than six residues on the carboxy-terminal side dramatically reduced the rate of peptide phosphorylation, but

shortening the sequence on the amino-terminal side actually increased the rate. Changing the glutamine (Q) at the position corresponding to p53 residue 16 to tyrosine (Y), asparagine (N), or lysine (K) decreased the rate of peptide phosphorylation, and inverting the glutamine and the following glutamic acid (SQE -> SEQ) essentially abolished peptide phosphorylation. Km values for the peptide substrates varied from about 0.2 to 0.7 mM; these values are not remarkably good compared to peptide substrates for several other kinases (Kemp and Pearson, 1991). A glutamine or glutamic acid immediately before the SQ motif and a glutamic acid immediately following it (i.e. QSQE or ESQE, as in the c-Jun site, see above) gave a slight improvement in the apparent association constant (compare peptides 4, 11, and 15 in Table 2); about a twofold further improvement was obtained by removing all but two residues from the amino-terminal side of the phosphorylation site (Table 1, Figure 1). We also noticed that the version of peptide 4 (EPPLSQEAFADLWKK) ending with an amide is a slightly better substrate than the same peptide ending with a carboxyl group, perhaps suggesting that the carboxy-terminal extension of six residues is not quite optimal. To date, the best substrate peptide for human DNA-PK is PESQEAFADLWKK, while the similar peptide PESEQAFADLWKK is not appreciably phosphorylated (Table 1, Figure 1).

A second substrate determinant for recognition by DNA-PK is the ability to bind DNA. Jackson et al. first observed that quantitative phosphorylation of Sp1 required a DNA template with a GC-box DNA binding element (Jackson et al., 1990).

Subsequently it was found that efficient phosphorylation required that both DNA-PK and Sp1 be bound to the same DNA molecule (Gottlieb and Jackson, 1993). Similar findings for murine p53 and a protein containing the POU DNA binding domain of human Oct-1 were made by Lees-Miller and Anderson (Lees-Miller et al., 1992; Anderson and Lees-Miller, 1992). DNA both activates DNA-PK and increases the local concentration of substrate in the vicinity of activated kinase. Short duplex oligonucleotides that bound kinase or the substrate but could not bind both simultaneously gave much lower rates of phosphorylation for p53 or the POU domain protein than long DNA fragments; however, the rates of phosphorylation of peptide substrates or of hsp90, a protein that does not bind DNA, were independent of DNA length or concentration. Thus, DNA length (above 18 bp) was not affecting kinase activation, nor was the interaction of the substrates with non-specific DNA sequences changing their conformation in a manner that significantly affected the rate of phosphorylation.

It seems likely that DNA binding also may be important for substrate recognition in vivo. Many site-specific DNA binding proteins, including p53 and SV40 TAg, bind DNA in a non-sequence-specific manner, and this ability may help proteins scan chromatin for their sequence-specific recognition sites. Presumably activated DNA-PK is fixed in space by binding to chromatin, although it may be able to slide in either direction from the initial binding site. Thus, we imagine that substrates become phosphorylated as they scan along chromatin strands via their non-sequence specific DNA binding mode and collide with

DNA-PK. Since both substrate and kinase will be physically constrained if both are associated with the same chromatin segment, the specific location of potential sites within the three dimensional structure of the substrate also may be important. Most proteins that function as DNA-PK substrates in vitro are DNA binding proteins (Anderson, 1993). If DNA binding ability accounts for a substantial fraction of substrate specificity and recognition, then DNA-PK may not have a highly specific sequence or structure recognition ability. This situation would account for the relatively poor K_m values that have been measured for substrate peptides.

If chromatin binding plays an important role in DNA-PK substrate recognition, then reaction conditions in the test tube are likely to be far removed from those within the nucleus of a cell. Thus, some putative substrates or sites that are phosphorylated in vitro may not be phosphorylated in vivo. One apparent example is the Oct-1 POU expression construct mentioned earlier. This protein (T7HPOU1) consists of the POU domain from human Oct-1 with a 17 amino acid amino-terminal extension derived from vector sequences (Figure 2). The POU domain has two sites that resemble other identified DNA-PK phosphorylated sites, a TQ at residues 44/45 and an SQ at residues 61/62. Phosphoamino acid analysis revealed only phosphoserine (data not shown); thus, the TQ sequence is not a DNA-PK phosphorylation site. After digestion with trypsin and CNBr, none of the phosphopeptides were retained on a C18 reverse phase column, but after digestion with trypsin alone, a major, modestly hydrophobic phosphopeptide was

retained by the reverse phase column (Figure 3). Amino-terminal sequence analysis showed that this peptide was derived from the amino terminus of the expressed protein and that the serine at position 3 was partially phosphorylated. These findings are consistent with the HPLC data shown in Figure 3; after digestion with CNBr and trypsin, serine 3 would be in the tripeptide Ala-Ser-Met* (Met* = homoserine). We cannot formally exclude serine 22 as a possible second site of phosphorylation; after digestion with both CNBr and trypsin, it would be present in the tetrapeptide Ser-Gly-Gly-Met*. However, another Oct-1/POU derivative containing serine 22 but lacking the serine 3 site was not phosphorylated by DNA-PK. Serine 3 lies outside the POU domain and is not followed by a glutamine, but, in this case, the non-specific DNA binding ability of the POU domain may be sufficient to drive phosphorylation of this non-physiological, vector-encoded site. Distinguishing between physiological and non-physiological substrates and sites may well be even more difficult for DNA-PK than for other protein kinases.

Our results with the POU domain protein raise the issue of whether in vivo DNA-PK also phosphorylates serines or threonines that are not followed by glutamine. One such putative target is the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II. The CTD consists of about 50 conserved repetitions of the consensus heptad motif YSPTSPS, and, although the CTD contains no SQ or TQ sites, it is phosphorylated efficiently by DNA-PK when coupled to a GAL4 DNA binding domain (Peterson et al., 1992). The CTD also becomes hyper-

phosphorylated in vivo during the initiation of transcription, and several putative CTD kinases have been described (Dhamus and Dynan, 1992; O'Brien et al., 1994).

A CTD peptide consisting of four heptad repeats, (YSPTSPS)₄RRR, is phosphorylated by purified human DNA-PK, although the K_m for this peptide substrate is above 1 mM. The CTD peptide was phosphorylated exclusively on serine (data not shown), and sequence analysis (Figure 4) revealed that the major site of phosphorylation was the serine at position 7, or the equivalent serine in the second (and probably at the third and fourth) repeat. These serines are followed in the repeat by tyrosine; however, tyrosine did not efficiently substitute for glutamine in the p53-derived sequence (Table 2). The GAL4-CTD protein was reported to be phosphorylated at both serine and threonine (Dvir et al., 1993); thus, the number of repeats, the DNA template, or other factors may influence the recognition of sites by DNA-PK.

Another consideration with regard to substrate recognition is that some sites phosphorylated in vivo may not be phosphorylated in vitro. We alluded to a possible case in SV40 TAg. Serine 639 is phosphorylated in vivo and resembles other DNA-PK sites, but no kinase has been identified that phosphorylates this site in vitro (Chen et al., 1991). In vivo TAg assembles into a dodecameric structure at the SV40 origin of replication, whereas, in our in vitro experiments, calf thymus DNA was used as the DNA-PK activator, and TAg most likely binds

in a non-sequence specific manner to fragments of calf thymus DNA primarily as monomers. It remains to be determined if the pattern of TAG residues phosphorylated by DNA-PK would be different if the Tag had been assembled on replication origins.

Conclusions. Most of the DNA-PK in normal, unsynchronized, cultured human fibroblasts and lymphoid cells is not tightly associated with DNA; thus, presumably it is inactive (Anderson and Lees-Miller, 1992). In vitro assays indicated that total cellular DNA-PK activity changes only slightly with stage of the cell cycle, and no significant increase in activity was observed after HeLa cells were treated with DNA damage-inducing agents. These observations suggest that DNA-PK normally may be inactive and that synthesis and degradation are not important mechanisms of regulating its activity. We presume that DNA-PK activation occurs as a consequence of changes to chromatin that create entry sites for the Ku DNA-binding, regulatory subunit. Such changes may be caused by normal cellular activities including transcription, replication or recombination, or they may be a consequence of damage to DNA that is caused by endogenous or exogenous agents. Further regulation may occur through autophosphorylation (Lees-Miller et al., 1990), other posttranslational events, or through the synthesis or activation of inhibitors (An et al., 1994). Because the methods used to disrupt cells in preparing extracts inevitably fragments chromatin, it has not been possible to determine the state of activation of DNA-PK with existing in vitro assays. Furthermore, because decay of the ^{32}P -label required to detect substrate

phosphorylation in vivo also produces DNA strand breaks that may activate DNA-PK, it has not been possible to correlate changes in the phosphorylation of putative substrates with kinase activation. Thus, a major challenge will be to develop assays suitable for measuring DNA-PK activity and activation in vivo. A detailed knowledge of the factors that affect DNA-PK substrate recognition in vitro undoubtedly will be helpful for the developing improved assays for DNA-PK activity.

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TABLE 1

Protein Phosphorylation Sites Recognized by Human DNA-PK

Substrate Protein ^a	DNA-PK Site	Local Amino Acid Sequence
		-*-
Hsp90[alpha] (human)	Thr4	P-E-E-T-Q-T-Q-D-Q-P-M-E-E ¹³
Hsp90[alpha] (human)	Thr6	P-E-E-T-Q-T-Q-D-Q-P-M-E-E-E ¹⁵
SV40 Large Tumor Antigen	Ser120	E-A-T-A-D-S-Q-H-S-T-P-P-K-K-K ¹²⁹
SV40 Large Tumor Antigen	Ser665	E-T-G-I-D-S-Q-S-Q-G-S-F-Q-A-P ⁶⁷⁴
SV40 Large Tumor Antigen	Ser667	G-I-D-S-Q-S-Q-G-S-F-Q-A-P-Q-S ⁶⁷⁶
SV40 Large Tumor Antigen	Ser677	Q-A-P-Q-S-S-Q-S-V-H-D-H-N-Q-P ⁶⁸⁶
c-Jun Transcription Factor (human)	Ser249	P-I-D-M-E-S-Q-E-R-I-K-A-E-R-K ²⁵⁸
Serum Response Factor (human)	Ser435	V-L-N-A-F-S-Q-A-P-S-T-M-Q-V-S ⁴⁴⁴
Serum Response Factor (human)	Ser446	M-Q-V-S-H-S-Q-V-Q-E-P-G-G-V-P ⁴⁵⁵
p53 Tumor Suppressor (mouse)	Ser4	M-E-E-S-Q-S-D-I-S-L-E-L-P ¹³
p53 Tumor Suppressor (mouse)	Ser15	L-E-L-P-L-S-Q-E-T-F-S-G-L-W-K ²⁴
p53 Tumor Suppressor (human)	Ser15	V-E-P-P-L-S-Q-E-T-F-S-D-L-W-K ²⁴
		-*-

^aPhosphorylation sites (-*-) were identified by: hsp90, Lees-Miller and Anderson (1989a); SV40 TAg, Chen et al. (1991); c-Jun, Bannister et al. (1993); serum response factor (SRF), Liu et al. (1993), and p53 (Lees-Miller et al. (1992).

TABLE 2

Phosphorylation of Synthetic p53 Peptide Substrates by Human DNA-PK

Pept. No.	Peptide Sequence										Apparent Km (mM)	Apparent Vmax (nmol/min/mg)	Activity PO ₄ /min/mg at 0.2 mM
p53	11	15	20	24									
1	E P P L S Q E T F S D L W K-K										0.35	360	130
2	- - - - S Q - - - KK										ND	ND	15
3	- - - - S Q - - - - - KK										ND	ND	12
4	- - - - S Q - A - A - - - - K										0.76	380	83
5	- - - - S Q - A - A - - L - K										0.56	160	36
6	- - - - S Y - A - A - - L - K										ND	ND	2.3
7	- - - - S E - A - A - - - - K										ND	ND	0.4
8	- - - - S N - A - A - - - - K										ND	ND	0.1
9	- - - - S E Q A - A - - - - K										ND	ND	0
10	- - - - S Q K A - A - - - - K										1.0	70	~14
11	- - - Q S L - A - A - - - - K										0.4	310	99
12	- - - Q S Q - A - A - - - - K										0.3	390	161
13	- - - T Q - A - A - - - - K										0.67	460	116
14	- - - D S Q - A - A - - - - K										ND	ND	48
15	P E E S Q - A - A - - - - K										0.27	410	180
16	P E S Q - A - A - - - - K										0.20	470	220
17	P E S E Q A - A - - - - K										ND	ND	0

Dash (-) indicates amino acid is same as in control peptide #1, p53(11-24)K; ND = not determined. Phosphorylation reactions were performed as described in Lees-Miller et al. (1992).

FIGURES

Figure 1. Phosphorylation of synthetic peptides by human DNA-PK.

Rates of peptide phosphorylation are shown as a function of peptide concentration. Reactions were at 30°C for 10 min as described (Lees-Miller et al., 1992); the data represent an average of three independent experiments for each peptide. Apparent Km and Vmax values were determined from Lineweaver-Berk plots. These values were respectively: (a) PESQEAFADLWKK_{COOH}, 0.20 mM, 470 nmol/min/mg; (b) PEESQEAFADLWKK_{COOH}, 0.27 mM, 410 nmol/min/mg; (c) PEESQEAFADLWKK_{amide}, 0.4 mM, 370 nmol/min/mg. Note that the Km value for (c) is about half that of its carboxyl group terminated equivalent peptide (Table 2, peptide 4). Peptides EPPLSEQAFDLWKK and PESEQAFADLWKK were not significantly phosphorylated (data not shown).

Figure 2. Sequence of the Oct-1/Pou expression construct

T7HPOU1. The predicted amino acid sequence of the human Oct-1 POU-domain construct from plasmid pT7HPOU1 (Anderson and Lees-Miller, 1992) is given in the single letter code. The POU domain extends from residue 19 to 178; residues 1 to 18 are derived from vector sequences and include a six-histidine tag for affinity purification. The POU domain has a TQ motif at residues 44/45 and a SQ motif at residues 61/62 (bold). Serine 3 (bold) is phosphorylated by DNA-PK (see text); the initiating methionine (residue 1) is removed in E. coli

Figure 3. Reverse phase fractionation of phosphopeptides from the Oct-1 POU domain polypeptide after phosphorylation by human DNA-PK. Purified Oct-1/POU-domain construct (T7HPOU1, see Figure 3) was incubated with DNA-PK, calf thymus DNA, and [^{32}P]ATP as described (Anderson and Lees-Miller, 1989b), digested with trypsin and CNBr (solid line) or with trypsin alone (dashed line), and the resulting peptides were fractionated by reverse phase (C18) HPLC essentially as described (Chen et al., 1991).

Figure 4. Sequence analysis of an RNA polymerase II CTD peptide phosphorylated by human DNA-PK. Top: The RNA polymerase II CTD peptide (YSPTSPS)₄RRR, containing 4 heptad repeats, was labeled by incubation at 1 mM with purified human DNA-PK, calf thymus DNA, and [^{32}P]ATP. After desalting on BioGel P4, the labeled peptide (100,000 cpm) was applied to a Beckman 890M sequencer together with polybrene (3 mg) and apomyoglobin (0.1 mg). The radioactivity released after each Edman cycle is shown; the peptide sequence is given at the top of the figure. The major sites of phosphorylation were serines 7, and 14 (*). A substantial lag in the release of phosphate occurs with this method of sequence analysis. Bottom: The CTD peptide was phosphorylated as described above, and the phosphorylated peptide was enriched by iron-affinity chromatography (Lees-Miller and Anderson, 1989a). Before sequence analysis, phosphoserine was

converted to S-ethylcysteine (SEC) by incubation with ethanethiol in 0.1 M barium hydroxide (Lees-Miller et al, 1989a). Shown are limited regions of the PTH-amino acid chromatogram near the diphenylthiourea (DPTU) peak from sequencer cycles 1, 2, 5, 7, and 9; SEC elutes immediately before DPTU. An Applied Biosystems 470A protein sequencer equipped for on-line PTH detection was used for this analysis.

Figure 1

Phosphorylation of Synthetic
Peptides by Human DNA-PK

FIGURE 2

	5	10	15	20	25	30
1	M A S M T G H H H H H H G M S G G M E E P S D L E E L E Q F					
31	A K T F K Q R R I K L G F T Q G D V G L A M G K L Y G N D F					
61	S Q T T I S R F E A L N L S F K N M C K L K P L L E K W L N					
91	D A E N L S S D S S L S S P S A L N S P G I E G L S R R R K					
121	K R T S I E T N I R V A L E K S F L E N Q K P T S E E I T M					
151	I A D Q L N M E K E V I R V W F C N R R Q K E K R I N P *					

Figure 3

Figure 4