pose, the duplex was added as a potential inhibitor of the aminoacylation of $tRNA^{Ala}$. At pH 7.5, no inhibition of aminoacylation was observed with a 50-fold excess of the I3·U70/G4·C69 RNA duplex. Therefore, although removal of the 2-amino group from position 3 of the duplex reduces the overall rate of aminoacylation (k_{cat}/K_m) to no more than 1/600 of the original rate, we estimate that the enzyme-RNA dissociation constant (K_m) is increased by at least 20-fold.

Transfer of the G3·U70 base pair into other tRNAs or into minihelices that are based on the sequences of other tRNAs results in aminoacylation with Ala (*3, 4, 14*). Although the N73 nucleotide (*14*) and the N2·N71 base pair (*15*) in particular modulate aminoacylation efficiency, no unique nucleotide or base pair at these positions is required for aminoacylation of the G3·U70-containing substrates. Thus, the exocyclic 2-amino group of G3·U70 is recognized by the enzyme in the context of different sequence variants in the acceptor stem. However, all of these variants presumably adopt the A-form helix where the 2-amino group is accessible from the minor groove. In view of the results reported here, the observation of the failure to aminoacylate a DNA helix (3′-rA) whose sequence is based on the acceptor stem of $tRNA^{Ala}$ (*6*) might be explained by its B-form geometry, where the 2-amino group in the minor groove is substantially less accessible than in the A-form structure (*1, 2*).

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Variable Effects of Phosphorylation of Pit-1 Dictated by the DNA Response Elements

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Pit-1, a tissue-specific POU domain transcription factor, is required for the activation of the prolactin, growth hormone, and Pit-1 promoters that confer regulation by epidermal growth factor, adenosine 3′,5′-monophosphate (cAMP), and phorbol esters. Pit-1 is phosphorylated in pituitary cells at two distinct sites in response to phorbol esters and cAMP. Phosphorylation of Pit-1 modifies its conformation on DNA recognition elements and results in increased binding at certain sites and decreased binding at other sites, dependent on DNA sequences adjacent to the core Pit-1 binding motif. One residue (Thr^{220}), located in the POU homeodomain within a sequence conserved throughout the POU-domain family, confers these responses.

PIT-1 APPEARS TO SERVE AS THE PITUITARY-specific activator of the growth hormone and prolactin genes during normal ontogeny (*1–6*) and is necessary for the development and proliferation of lactotroph, somatotroph, and thyrotroph cells in the anterior pituitary gland. Pit-1 autoregulates its own gene (*7, 8*). Its binding sites have been implicated in the activation of the growth hormone gene by cAMP (*9*) and the transcriptional regulation of the rat prolactin gene by cAMP, epidermal growth factor (EGF), and phorbol esters (TPA) (*10*). EGF, cAMP, and TPA, which are known to activate specific protein kinases, thus, might exert their effects on prolactin and growth hormone gene expression by way of phosphorylation of Pit-1.

Immunoprecipitation from a pituitary cell line (GC) labeled in vivo revealed that the amount of ^{32}P label in both the 33- and 31-kD Pit-1 variants (*11*) increased four- to eightfold in response to dibutyryl cAMP and three- to sixfold in response to TPA. The rate of synthesis of Pit-1, quantitated by [^{35}S]methionine labeling (*12*), was equivalent in control and treated cells (Fig. 1). After stimulation, >80% immunoprecipitated Pit-1 migrated as phosphoprotein by isoelectric focusing (*13*). Treatment with Co^{2+}, which inhibits the activation of protein kinase C and prolactin gene expression in GC cells (*14*), decreased basal Pit-1 phosphorylation twofold. Because of the prolonged exposure of Pit-1 to cellular phosphatases during immunoprecipitation, the extent of phosphorylation is likely to be underestimated. Two-dimensional (2D) chromatographic patterns of tryptic phosphopeptides generated from Pit-1 suggested that the sites of phosphorylation were similar in control, dibutyryl cAMP–, or TPA-

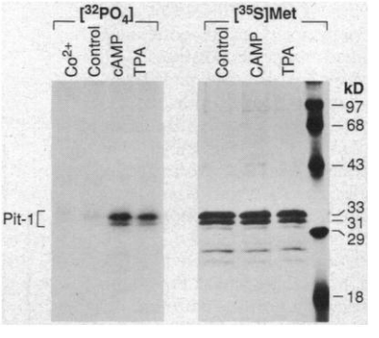

Fig. 1. Phosphorylation of Pit-1 in response to cAMP and phorbol esters. SDS-PAGE autoradiograms show [^{32}P]orthophosphate- and [^{35}S]methionine-labeled Pit-1 immunoprecipitated from GC cells that were unstimulated (control), TPA- or cAMP-induced, or treated with $CoCl_2$.

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*The contribution of M.S.K. and Y.F. to this manuscript were equivalent and both authors should be considered as primary authors.

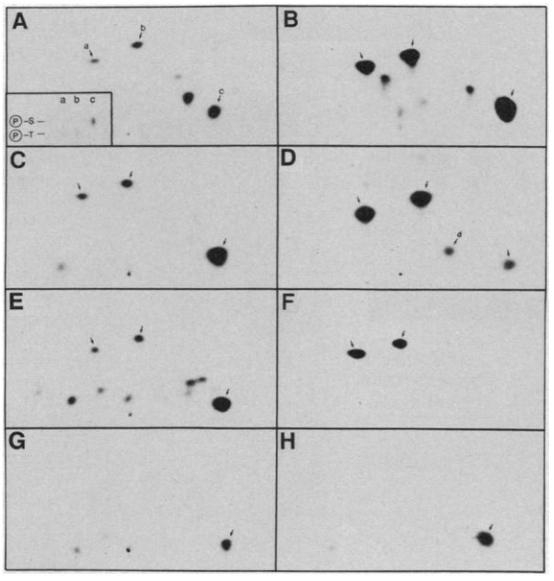

Fig. 2. Two-dimensional tryptic phosphopeptide mapping of phosphorylated Pit-1. Pit-1 was isolated from unstimulated (**A**), TPA-treated (**C**), or cAMP-treated (**E**) GC cells. Bacterially expressed wild-type and mutant Pit-1 proteins (see text) were phosphorylated in vitro by protein kinase C (**D**) or by protein kinase A [wild type (**B**), mutant A115 (**F**), mutant A 219,220 (**G**), and mutant A220 (**H**)]. The major tryptic peptides are marked by arrows (a through c mark peptides phosphorylated both in vivo and in vitro and d marks an additional peptide phosphorylated only by protein kinase C in vitro). Unmarked peptides are partial proteolytic products. Equivalent amounts of radioactivity were used. (**Inset**) High-voltage electrophoresis of acid hydrolysates and comparison to migration of phosphoamino acid standards showed that peptides a and b contained phosphothreonine, and peptide c contained phosphoserine.

treated cells (Fig. 2) (*15*).

Bacterially expressed Pit-1 (*15*) was incubated in vitro with various purified protein kinases. Protein kinases A and C, but neither casein kinase II nor type II calcium-calmodulin–dependent protein kinase phosphorylated Pit-1. Approximately 1 to 2 moles of phosphate were incorporated per mole of Pit-1. The pattern of tryptic peptides obtained after in vitro labeling of Pit-1 with protein kinase A was similar to that observed in vivo (Fig. 2), as confirmed in cochromatography experiments. The sites of phosphorylation were tentatively assigned to Thr^{219} or Thr^{220} in the homeodomain and to Ser^{115} by sequence analysis of V8 *Staphylococcus aureus* and tryptic proteolytic phosphopeptides obtained from Pit-1 phosphorylated in vitro (Fig. 3) (*16*).

Pit-1 proteins with alanine substitutions at residues 115, 219, or 220 were generated. After high-performance liquid chromatography (HPLC) separation of the V8 proteolytic phosphopeptides from a mutant with a substitution at position 115 (A115), only peptide 1 was detected (Fig. 2). Conversely mutation of positions 219 and 220 (A219,220) led to the loss of phosphorylated peptide 1. A triple mutant with alanine residues at positions 115, 219, and 220 (A-3) was no longer a substrate for protein kinase A (*17*). The predominant site of homeodomain phosphorylation was identified as Thr^{220} (Fig. 3). Phosphorylation of Thr^{219} by protein kinase A or C was observed only after extended incubation in the A220 mutant (Thr^{220} to Ala), whereas phosphorylation of Thr^{220}, in the context of the A219 mutant (Thr^{219} to Ala), proceeded as rapidly as in wild-type Pit-1. Tryptic map analysis of the Pit-1 mutants permitted assignment of the three major tryptic peptides to Ser^{115} and Thr^{220} in vitro (Fig. 2). It is likely, although not proven, that these residues are those phosphorylated in vivo.

After phosphorylation, Pit-1 exhibited decreased (10-fold to 20-fold) binding affinity for the growth hormone element GH-1, slightly lower affinity for the prolactin 1P binding site (one- to twofold), no change in affinity for the positive 5′ *pit-1* gene autoregulatory site (PB-1), and a two- to threefold increased affinity for the proximal prolactin promoter 3P site (Fig. 4A) (*18*). In addition, Pit-1 phosphorylation increased dimerization of Pit-1 on the growth hormone element GH-2 (Fig. 4A) and on the prolactin 3D site, decreased the binding affinity for the prolactin enhancer sites 1D, 2D, and 4D, and had little effect on the affinity for the negative *pit-1* gene site (PB-2) (*19*). Effects were independent of basal binding affinity. Only mutation of Thr^{220} (mutants A220 and A-3) (Fig. 4B) abolished the phosphorylation-related decrease in affinity for the GH-1 site. The binding characteristics of unphosphorylated Pit-1 were not altered by any of the introduced mutations as assayed by gel shift or DNA footprinting analyses.

Using chimeras, we identified the sequences determining the inhibitory effects of phosphorylation on Pit-1 binding to GH-1, 6 to 12 base pairs (bp) 5′ of the Pit-1 TATNCAT binding consensus sequence. The chimeric sites that failed to exhibit the inhibitory effects of phosphorylation on Pit-1 binding contained an ATTA sequence in this region (Fig. 4C). No single base alone could account for the difference in responsiveness to phosphorylation.

In methylation interference studies (*18*), modification of two guanidines, 5′ to and within the TATNCAT sequence of the PB-1 site, was found to impede binding of unphosphorylated Pit-1 (Fig. 4D), indicating that unphosphorylated Pit-1 contacted two adjacent major grooves on the same face of the DNA duplex. Binding of phosphorylated Pit-1, in contrast, was only susceptible to methylation of the guanidine in the consensus sequence (Fig. 4D). This suggested that

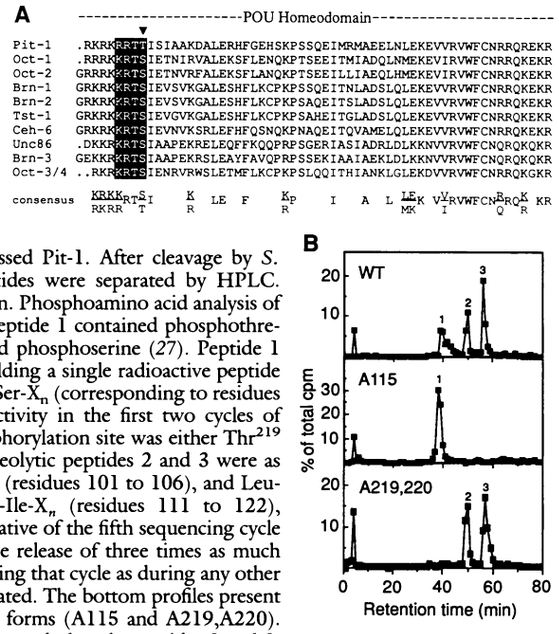

Fig. 3. Sites of Pit-1 phosphorylation. (**A**) The threonine phosphorylation site is localized within a sequence conserved throughout the POU-domain family (*22*). POU homeodomain sequences are shown, with the phosphorylation site in reverse type and the residue phosphorylated in Pit-1 indicated by the arrowhead (*26*). (**B**) Determination of protein kinase A phosphorylation sites of bacterially expressed Pit-1. After cleavage by *S. aureus* V8 protease, proteolytic peptides were separated by HPLC. Radioactive elution patterns are shown. Phosphoamino acid analysis of wild-type Pit-1 (WT) revealed that peptide 1 contained phosphothreonine and peptides 2 and 3 contained phosphoserine (*27*). Peptide 1 was further digested with trypsin, yielding a single radioactive peptide on HPLC with the sequence X-X-Ile-Ser-X_n (corresponding to residues 219 to 220), which released radioactivity in the first two cycles of sequencing. This suggested the phosphorylation site was either Thr^{219} or Thr^{220}. The sequences of V8 proteolytic peptides 2 and 3 were as follows: Asp-Pro-Thr-Ala-Ser-Glu-X_n (residues 101 to 106), and Leu-Arg-Arg-Lyx-X-Lys-Leu-Val-Glu-Pro-Ile-X_n (residues 111 to 122), respectively. Although the PTH derivative of the fifth sequencing cycle was not identifiable for peptide 3, the release of three times as much radioactivity in the column eluant during that cycle as during any other suggested that Ser^{115} was phosphorylated. The bottom profiles present the elution patterns for mutant Pit-1 forms (A115 and A219,A220). Mutation of Ser^{115} to Ala (A115) removed phosphopeptides 2 and 3.

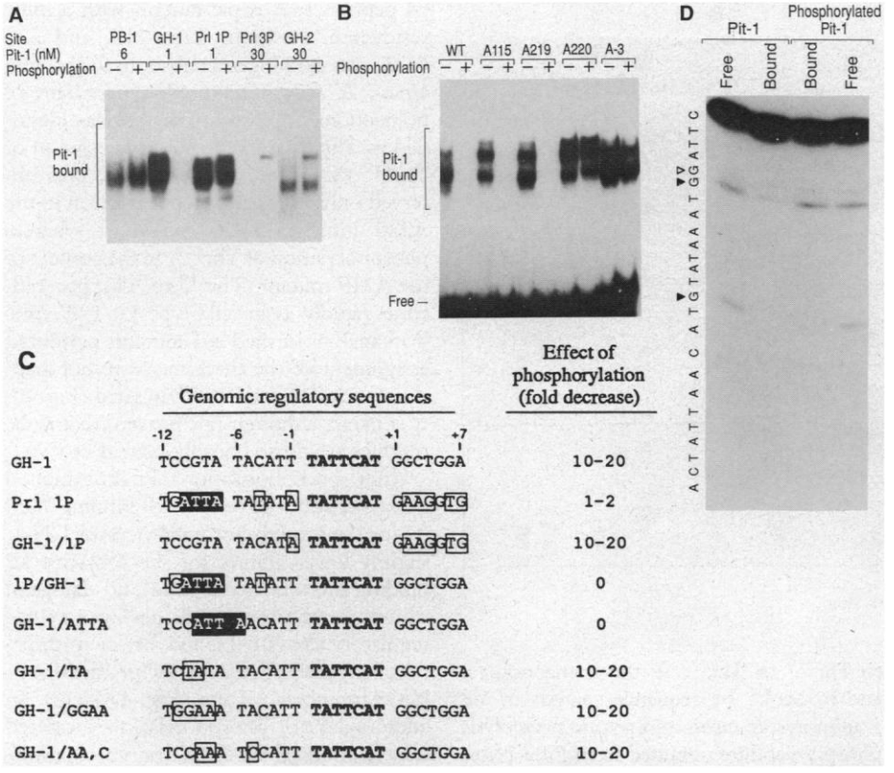

Fig. 4. Pit-1 binding and conformation on DNA are affected by phosphorylation. (**A**) Gel shift assays of Pit-1 binding to cis-active elements before (−) and after (+) phosphorylation. The amount of Pit-1 varied depending on the affinity of Pit-1 for that site. Excess oligonucleotide was used. (**B**) Unphosphorylated (−) and phosphorylated (+) Pit-1 mutants containing Ala substitutions for one (A115, A219, and A220) or all (A-3) potentially phosphorylated residues were assayed for the ability to bind the GH-1 site. (**C**) Effect of phosphorylation on Pit-1 binding affinity to modified GH-1 sites. GH-1/1P and 1P/GH-1 are chimeras of the GH-1 and Prl 1P sites. The GH-1/ATTA, GH-1/TA, GH-1/GGAA, and GH-1/AA,C sites represent substitutions of the GH-1 site with sequences from the PB-1 site. (**D**) Major groove contacts on PB-1 were determined in methylation interference experiments. One of the contacts (solid arrowheads) disappeared concomitant with phosphorylation. The open arrowhead marks a guanidine not subject to interference.

phosphorylation alters the conformation of Pit-1 on DNA.

Posttranslational modification of transcription factors by phosphorylation affects transcription through diverse mechanisms, including modulation of nuclear translocation (*20*) and activation of constitutively bound transcription factors (*21, 22*). A third mechanism involves the regulation of DNA binding by phosphorylation (*23*). The identification of the phosphorylation site on Pit-1 and the nucleotides in its recognition elements that dictate the effects of phosphorylation shows that the sequences adjacent to a core binding motif may impose specific, qualitatively distinct responses to phosphorylation of a residue within the DNA binding interface of a transcription factor. These responses include increased or decreased binding of Pit-1 to various promoter elements and altered Pit-1 conformation on DNA binding. The altered conformation can increase DNA-dependent dimerization of Pit-1 (Fig. 4A) and could result in differential presentation of the transactivation domain to the cellular transcriptional apparatus at specific sites. Because of the differential effects of Pit-1 phosphorylation on the many recognition elements within the promoters studied here, the precise role of Pit-1 phosphorylation in the transcriptional regulation of these genes will require analysis of its combinatorial actions on multiple regulatory elements. The events described for Pit-1 may be prototypic because the phosphorylation site $(K/R)_4$ RT (S/T) I is conserved between the homeodomains of other POU-domain proteins (*24, 25*).

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16. After phosphorylation of purified, bacterially expressed Pit-1 with protein kinase A (*15*), each sample was purified by SDS-PAGE. Peptides from *Staphylococcus aureus* V8 digestions in 0.5 M guanidine, 25 mM Na_2HPO_4 (pH 7.8), were separated by C4 column reverse-phase HPLC [D. A. Bullough and W. S. Allison, *J. Biol. Chem.* **261**, 5722 (1986)]. Sequencing of peptides was performed on an Applied Biosystems 470A gas phase sequencer with the standard 03RPTH program supplied by the manufacturer and with phenylthiohydantoin derivatives analyzed by a model 120 on-line HPLC. Pit-1 mutants were constructed by site-directed mutagenesis (*4*).
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18. Phosphorylation (*15*) was performed with the use of nonradioactive ATP. Pit-1 perturbed gel shift analyses were performed with protein kinase A. Several experiments with protein kinase C suggested similar effects on Pit-1 binding. Binding was performed with diluted kinase reaction and 0.3 nM end-labeled oligonucleotide probe (GH-1; −95 to −70 bp; GH-2; −144 to −106 bp; PB-1; −65 to −40 bp; Prl-1P; −66 to −38 bp; Prl-3P; −168 to −140 bp) as described (*4*). Changes in affinity were quantitated with the use of an Ambis two-dimensional scintillation detector. Saturation curves were generated for binding of unphosphorylated and phosphorylated Pit-1 by variation of the oligonucleotide concentration. We determined 50% saturation points and used them to calculate binding affinities, assuming a trimolecular reaction. Because of the inherent error in quantitating gel shift experiments, binding could only be approximately estimated. The data were quantitated in four separate experiments for each DNA element. For methylation interference experiments, the Pit-1 oligonucleotide probe (3 nM) was 5′ labeled at one end and methylated before incubation with Pit-1 (30 nM). Protein-bound and free probes were separated by PAGE and cleaved by piperidine, and equal amounts of radioactivity were analyzed on a 15% sequencing gel.
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26. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
27. M. S. Kapiloff, Y. Farkash, M. Wegner, M. G. Rosenfeld, unpublished data.
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Identification of Ets- and Notch-Related Subunits in GA Binding Protein

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Recombinant cDNA clones that encode two distinct subunits of the transcription factor GA binding protein (GABP) have been isolated. The predicted amino acid sequence of one subunit, GABPα, exhibits similarity to the sequence of the product of the *ets*-1 proto-oncogene in a region known to encompass the Ets DNA binding domain. The sequence of the second subunit, GABPβ, contains four 33–amino acid repeats located close to the NH_2-terminus of the subunit. The sequences of these repeats are similar to repeats in several transmembrane proteins, including Notch from *Drosophila melanogaster* and Glp-1 and Lin-12 from *Caenorhabditis elegans*. Avid, sequence-specific binding to DNA required the presence of both polypeptides, revealing a conceptual convergence of nuclear transforming proteins and membrane-anchored proteins implicated in developmentally regulated signal transduction processes.

HERPES SIMPLEX VIRUS 1 (HSV1) immediate early (IE) genes are induced at the outset of lytic infection by a virion-associated protein termed VP16 (*1*). At least two classes of cis-regulatory elements are required in HSV1 IE genes for induction by VP16. The most essential VP16 cis-response element is characterized by the nonanucleotide sequence 5′-TAATGARAT-3′ (*2, 3*). VP16 binds tightly to this DNA sequence in a complex with the cellular transcription factor Oct-1 (*4*). A second cis-regulatory element required for VP16-mediated induction of HSV1 IE genes consists of three imperfect repeats of the purine-rich hexanucleotide 5′-CGGAAR-3′ (*3, 5*). A protein complex capable of avid interaction with the purine-rich repeats (GA repeats) has been identified in soluble preparations of rat liver nuclei (*3*). This GA binding protein (GABP) consists of two separable subunits. Purified samples of either subunit do not interact with the GA repeats, yet regain potent DNA binding activity when mixed (*6*).

In an effort to resolve the molecular basis of subunit interdependency of GABP activity, we isolated recombinant cDNA clones of their encoding genes. GABP (20 μg) was purified from rat liver nuclear extracts and cleaved with trypsin. Proteolyzed fragments were separated by high-performance liquid chromatography (HPLC), recovered, and subjected to gas-phase amino acid sequencing (Fig. 1). Partial sequences were derived from 13 tryptic peptides. Degenerate oligonucleotides capable of encoding 4 of the 13 peptide sequences were synthesized and used as hybridization probes to screen an adipocyte cDNA library (7). One recombinant bacteriophage that contained a cDNA insert of 2 kb gave a positive signal when hybridized with each of the four oligonucleotide probes.

The insert of this recombinant was sequenced and found to contain an open reading frame that encoded a protein of 454 amino acids (Fig. 2A). The predicted molecular size of this polypeptide (51.3 kD) corresponded to the size of the α subunit of GABP (GABPα) purified from rat liver nuclei (*6*) (Fig. 1). Inspection of the deduced amino acid sequence revealed segments that corresponded to 8 of the 13 peptides isolated by trypsin digestion of intact GABP. On the basis of the latter two observations, we tentatively identified this 454-residue polypeptide as GABPα.

Degenerate oligonucleotides capable of encoding two of the tryptic peptide sequences not present in GABPα were synthesized and used as hybridization probes to search for a cDNA clone that encoded the β subunit of GABP (GABPβ) (*8*). Five recombinant bacteriophages were identified by their capacity to hybridize with both oligonucleotide probes. One of the cDNA clones differed at the 3′ end from the other four. The largest cDNA insert of the four (2.6 kb) and the variant (1.4 kb) were sequenced. Both DNA sequences revealed long open reading frames specifying very similar polypeptides (Fig. 2B). One cDNA encoded a protein of 382 amino acids, the other encoded a protein of 349 residues. Starting at their respective NH_2-termini, the two proteins exhibited identical sequences for 333 amino acids. At this point the sequences of the two proteins diverged such that the longer one contained an additional 50 residues before its COOH-terminus, and the other an additional 15 residues before its terminus. The divergent COOH-terminal segments bore no apparent amino acid sequence similarity. The open reading frames of both polypeptides contained segments that corresponded to the two tryptic peptides used to design hybridization probes. Moreover, the predicted molecular sizes of the two

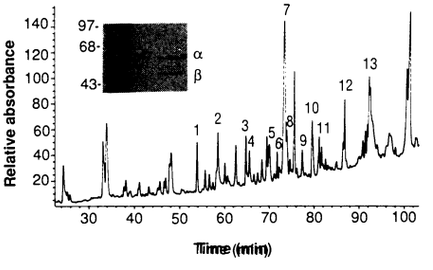

Fig. 1. Amino acid sequences of tryptic peptides derived from GA binding proteins. GABP (20 μg) was purified to homogeneity (inset) as described (*6*), except that boiled, salmon sperm DNA (20 μg/ml) was included as a nonspecific competitor in the DNA affinity chromatographic step. Approximately 500 pmol of protein was lyophilized, reduced, acetylated, and subject to cleavage by trypsin (Boehringer Mannheim). The resulting peptides were separated by reversed-phase HPLC (*22*). Amino acid sequence analysis was performed on a Vydac C18 column (*23*). The amino acid sequences derived from peaks 1 to 13 were 1, SLFDQGVIEK; 2, ?AWALEGY; 3, DEIS?VGDEGEFK; 4, ELESLNQEDFFQR; 5, LQESLDAHEIELQDIQL?P?R; 6, DQISIVGDEGEFK; 7, MAELV; 8, YVQASQLQQM- NEIVT-IDQP; 9, TPLHMWASEGHA; 10, GEILWS; 11, LIEIEIDGTEK; 12, ILMANGAPFTTD; 13, TGNNGQIQL?QFLLEL?TDR. Molecular size markers in kilodaltons are given at the left of the inset.

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