(1991) *Biochem. J.* (in press).
Welgus, H. G., Jeffrey, J. J., Eisen, A. Z., Roswit, W. T., & Stricklin, G. P. (1985) *Collagen Rel. Res. 5*, 167–179.
Wilhelm, S. M., Collier, T. E., Marmer, B. L., Eisen, A. Z., Grant, G. A., & Goldberg, G. I. (1989) *J. Biol. Chem. 264*, 17213–17221.
Williamson, R. A., Marston, F. A. O., Angal, S., Koklitis, P., Panico, M., Morris, H. R., Carne, A. F., Simth, B. J., Harris, T. J. R., & Freedman, R. B. (1990) *Biochem. J. 268*, 267–274.

Articles

A Pancreatic Exocrine Cell Factor and AP4 Bind Overlapping Sites in the Amylase 2A Enhancer[†]

E. Fodor,[‡] S. L. Weinrich,[‡] A. Meister,[‡] N. Mermod,[§,∥] and W. J. Rutter[*,‡]
Department of Biochemistry and Biophysics, Hormone Research Institute, University of California, San Francisco, California 94143, and Department of Biochemistry, University of California, Berkeley, California 94720
Received February 21, 1991; Revised Manuscript Received June 10, 1991

ABSTRACT: A factor found in pancreatic exocrine cell lines and pancreatic nuclei binds selectively to the α-amylase 2A transcriptional enhancer. Pancreatic exocrine cell extracts protect asymmetrically an unusually large, 35 base pair region from DNase I digestion in vitro, suggesting the involvement of a multimeric DNA binding complex. We show that this region of the enhancer contains a major affinity recognition sequence for the HeLa transcription factor AP4. A 4 base pair mutation in the enhancer sequence shown previously to abolish activity in vivo [Boulet, A. M., Erwin, C. R., & Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3599–3603] abolishes AP4 binding in vitro and weakens but does not eliminate the binding of adjacent enhancer factors. Further, sequences similar to the AP4 binding site are found within a consensus sequence of most pancreatic exocrine genes (Boulet et al., 1986). We have identified three AP4 binding sites in the pancreatic elastase gene: one occurs in the consensus sequence of the enhancer. Thus, protein(s) with the binding selectivity of AP4 may play a role in the expression of the pancreatic exocrine gene family.

Acinar cells of the exocrine pancreas produce and secrete hydrolytic enzymes which are involved in intestinal digestion. It is believed that the differentiated phenotype of this and other cells results from stringent control of activation and repression of gene sets by nuclear proteins which interact with DNA target sites in tissue-specific promoter and enhancer elements (Dynan & Tjian, 1985; Ptashne, 1988). Previous studies from this laboratory (Boulet et al., 1986) and others (Ornitz et al., 1985) have shown that high levels of transcription of many acinar cell-specific genes are determined by enhancer elements located in their 5′-flanking region.

The genes encoding the pancreatic hydrolytic enzymes contain a conserved 20 base pair sequence 100–200 base pairs from their mRNA CAP site (the pancreatic consensus sequence) (Boulet et al., 1986; Ornitz et al., 1985). This element appears essential for the activity of the amylase, chymotrypsin, elastase, and trypsin gene enhancers (Boulet et al., 1986; Hammer et al., 1987). The amylase 2A promoter–enhancer (+1 to −200 nucleotides from the mRNA CAP site) is necessary and sufficient to effect transcription of linked genes in cells of pancreatic exocrine origin but not in other cell types (Walker et al., 1983). In trangenic animals, this element has also been shown to be a determinant for the pattern of developmental expression of the amylase and elastase genes (Hammer et al., 1987; Osborn et al., 1988).

In the present work, we characterize by DNase I footprinting and electrophoretic mobility shift assays the binding in vitro of the amylase enhancer to nuclear factors present in amylase-expressing and -nonexpressing cell lines. The binding profile of the amylase enhancer is different from that of a mutant enhancer containing a 4 base pair transition mutation within the pancreatic consensus sequence that abolishes activity in vivo. We observed that the DNA sequence encompassing the region of the mutation is similar to the distal region of the mammalian virus SV40 late promoter that includes the binding site for transcription factor AP4 (Mermod et al., 1988). Experiments reveal that the enhancer binds purified AP4 with high affinity and that the 4 bp mutation eliminates binding activity. The pancreatic elastase 5′ region contains multiple AP4 binding sites. One of these sites is present within the pancreatic consensus element of this gene (Ornitz et al., 1985).

MATERIALS AND METHODS

DNA Probes. pAmy, containing the rat pancreatic α-amylase 2A promoter–enhancer (+30 to −350), was obtained from a partial digest of pAmy.CAT (Boulet et al., 1986) and inserted into the *Xho*I site of pUC9 by standard methods (Maniatis et al., 1982). The 57 base pair (−108 to −165) amylase minimal enhancer and enhancer mutant IV that contains a 4 base pair mutation (CAGT at −121 to −124) have been described (Boulet et al., 1986). The rat pancreatic elastase promoter–enhancer was obtained from R. MacDonald (Kruse et al., 1988) and the +8 to −205 *Sal–Bam* fragment subcloned into the polylinker of pUC19. A *Pvu*II–*Sph*I SV40

[†] N.M. was supported by a Swiss NSF postdoctoral fellowship. This work was supported by NIH Grant DK-21344.
[‡] University of California, San Francisco.
[§] University of California, Berkeley.
[∥] Present address: Institut de Biologie Animale, Universite de Lausanne, 1015 Lausanne, Switzerland.

0006-2960/91/0430-8102$02.50/0 © 1991 American Chemical Society

enhancer fragment from pLSVE (Nir et al., 1988) was subcloned into the polylinker of pUC18 as previously described (Fodor et al., 1988).

For labeling, plasmids were linearized 5′ or 3′ to the inserted DNA with the appropriate restriction enzyme, dephosphorylated with alkaline phosphatase, and rephosphorylated with [γ-^{32}P]ATP and polynucleotide kinase. The singly end-labeled insert was released by recleaving either 5′ or 3′ of the labeled end and isolated by polyacrylamide gel electrophoresis (Maxam & Gilbert, 1980). In some cases, DNA was labeled by end-filling using the appropriate α-^{32}P-labeled dNTP's and the Klenow fragment of DNA polymerase I (Maniatis et al., 1982).

Cells and Extract. Hamster insulinoma (HIT) (Walker et al., 1983) and rat azaserine-transformed pancreatic acinar (AR42J) (Jessop & Hay, 1980) cell lines were grown in roller bottle culture by the Cell Culture Facility, UCSF. Rat fibroblast (XC) or rat lymphoid B cells were grown in multiple T-150 culture flasks. Subconfluent culture cells were harvested by using glass beads for roller bottles or scraping for T-150 flasks and washed twice with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS). Prior to extraction, 4 packed cell volumes of ice-cold 60% PBS were added followed by 20-min incubation on ice. The swollen cells were then lysed by 8 strokes of a "B" Dounce homogenizer and extracts prepared according to the method of Manley (Manley et al., 1980) with the exception of substituting HEPES buffer for Tris throughout. The dialyzed extract was divided into aliquots, quickly frozen, and stored at −70 °C until use. Protein concentration was determined by the method of Bradford (1976) using a Bio-Rad protein assay kit.

Canine pancreas was obtained fresh from the Surgery Department, UCSF, trimmed of fat and connective tissue, quick-frozen in liquid nitrogen, and stored at −80 °C until use. Extracts were prepared by first crushing and quickly pulverizing frozen in a precooled (−80 °C) stainless-steel 2 L Waring blendor. The powdered tissue was slowly mixed with 100 mL of buffer A (20 mM HEPES, pH 8, 1 mM EDTA, 10 mM PMSF, and 20% glycerol) per gram of tissue and homogenized with a Kontes type B glass Dounce. The solution was filtered through three layers of cheesecloth and centrifuged at 2500g in a clinical centrifuge at 4 °C. The crude nuclei pellet was then washed 3 times by resuspension and brief Dounce homogenization and precipitation from ice-cold Mg^{2+}/Ca^{2+}-free PBS containing 10 mM PMSF. Protein was extracted from the nuclear pellet by the method of Manley (Manley et al., 1980) as described above.

DNase I Footprinting. Typically, 10 μL consisting of 1–4 μL of extract (4–10 mg/mL) diluted in dialysis buffer (Manley et al., 1980) was combined with 10 μL of DNA mix (5 mM Tris, pH 8, 5 mM NaCl, and 0.2 mg/mL carrier DNA [poly(dI-dC)] or sheared salmon sperm DNA) and incubated on ice for 10 min. One microliter of ^{32}P-labeled DNA probe was added (1–10 ng) and the mixture incubated on ice for 5 min. Samples were then warmed to 22 °C and incubated for 15 min. Digestion with DNase I and subsequent analysis were as described (Dynan & Tjian, 1983).

Gel Shift Assays. Cell extracts or chromatographic fractions were diluted with buffer A (20 mM HEPES, pH 8, 1 mM EDTA, and 20% glycerol) to an ionic strength of 0.1 M KCl, combined with an equal volume of DNA mix, and adjusted to a final $MgCl_2$ concentration of 5 mM and incubated on ice for 10 min. ^{32}P-Labeled DNA was then added and the reaction incubated for 5 min on ice. Samples were then placed at 22 °C for 15 min and carefully layered on a 4 or 6% polyacrylamide gel [30:1 acrylamide:bis(acrylamide) ratio] which was cast and prerun for 1 h in 10 mM Tris, pH 8, 3 mM NaAcOH, and 1 mM EDTA at 15 V/cm with buffer circulation (Carthew et al., 1985). Electrophoresis was carried out at 5 V/cm for 40 min after which the voltage was increased to 15 V/cm for approximately 2 h at 22 °C.

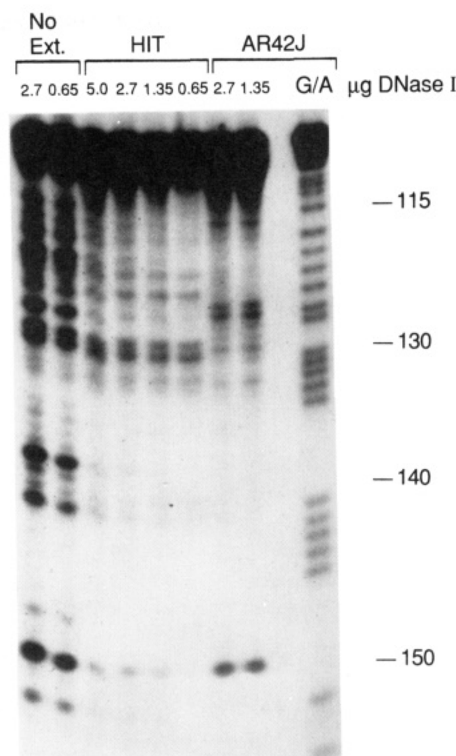

FIGURE 1: Comparison of the amylase 2A enhancer binding proteins in AR42J (6 mg/mL) and HIT (9 mg/mL) cell extracts. The 60 bp amylase enhancer was 5′-labeled on the coding strand and bound to AR42J or HIT cell extract (as above) or no protein (dialysis buffer) and digested with the indicated amounts of DNase I. A depurination of the probe is included as a size standard. Numbers are in bases from the amylase mRNA CAP site.

RESULTS

The Amylase Transcriptional Regulatory Region Forms a Complex with Exocrine Cell Factors in Vitro. To test whether the cis activity of the amylase 2A enhancer is correlated with a unique pattern of protein binding, we performed DNase I footprinting on the amylase enhancer in vitro using extracts from the amylase-expressing exocrine cell line AR42J (Jessop & Hay, 1980) and from the pancreatic endocrine tumor cell line HIT (Walker et al., 1983) that is negative for amylase expression.

A typical footprint of the amylase enhancer (−105 to −165 nucleotides from the mRNA CAP site) incubated in pancreatic cell extracts is presented in Figure 1. With AR42J extracts, protection from DNase I digestion occurred on the coding strand in two domains (from −110 to −130 and from −138 to −150), demarcated by DNase I hypersensitivity. With endocrine cell extracts, protection of the −110 to −130 region was clearly altered, with only weak binding in the −138 to −150 region.

An Amylase Enhancer Mutation Alters Binding to Exocrine Cell Proteins in Vitro. We tested whether an enhancer mutation that had a strong negative effect in vivo also affected the observed pattern of protein binding in vitro. Amylase mutant IV (−124TGTG−121 to CAGT) was used since it inactivates the enhancer in either orientation (Boulet et al., 1986) and occurs within the −110 to −130 region protected

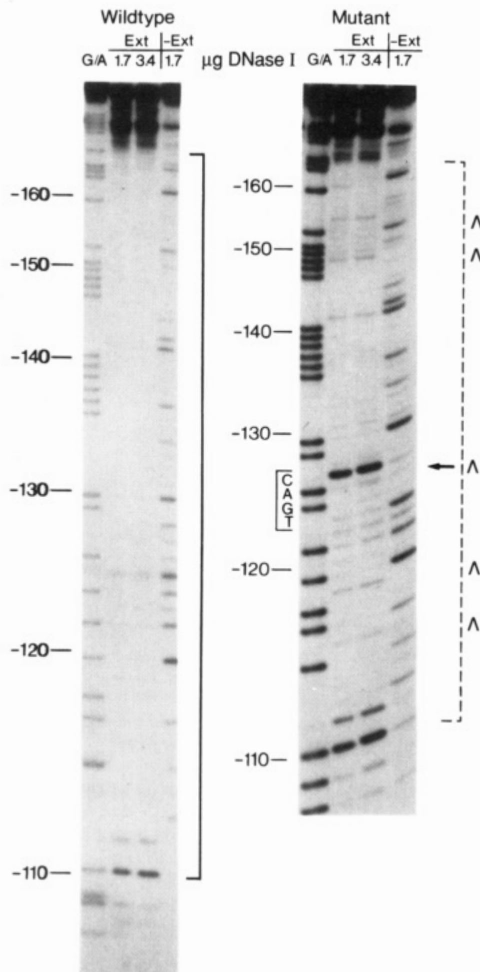

FIGURE 2: DNase I footprint of wild type and amylase mutant IV enhancer in AR42J whole cell extracts. (Left) Wild type or (right) mutant IV amylase enhancer (−105 to −165) 5′-labeled on the noncoding strand was incubated with AR42J cell extract and digested with DNase I as indicated. Brackets mark the limits of protection. Carets mark the presence of DNase I enhancements in the mutated sequence.

selectively by the exocrine cell extract.

In contrast to the coding strand (see Figure 1), the exocrine cell extract protected a broad domain on the noncoding strand from −110 to −160 (Figure 2, left). When a probe containing the amy IV mutant was used, a DNase I hypersensitivity was prominent within the bound region (Figure 2, right). A similar DNase I hypersensitivity was induced at the same position on the coding strand (data not shown). These data together with the comparable data in Figure 1 imply that the enhancer is occupied asymmetrically over a considerable region. Our results indicate that one effect of the strong inactivating mutation at −121 to −124 is to significantly alter the binding contacts in the DNA. However, the mutation does not appear to eliminate the binding of adjacent enhancer factors.

Specificity and Cell Type Distribution of the Amylase Enhancer Binding Protein. Electrophoretic mobility shift assays were employed to compare the affinity of the exocrine factor for the wild-type and mutated enhancer and to evaluate the distribution of the activity in other cell types. Cell extracts were first chromatographed on cellulose phosphate to concentrate the binding proteins and to provide a limited purification. Fractions were then bound to the labeled enhancer and electrophoresed through a 6% low ionic strength gel (Carthew et al., 1985). As shown in Figure 3, arrow, one or more factors from AR42J cells that bind to the amylase enhancer are retained by cellulose phosphate at 0.05 M KCl and

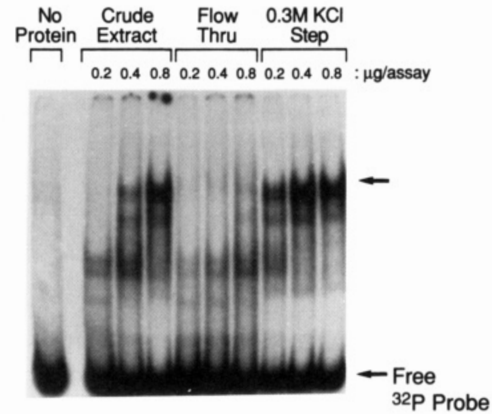

FIGURE 3: Cellulose phosphate chromatography of AR42J whole cell extracts. One milliliter of an AR42J whole cell extract was diluted to 50 mM KCl with buffer A (final protein concentration 2.3 mg/mL) and applied to a 1-mL column of cellulose phosphate equlibrated in buffer A at 50 mM KCl. After re-application, the column was washed with 10 column volumes of buffer A at 50 mM KCl. Protein was step-eluted with 0.3 M KCl in the same buffer, and 0.5-mL fractions were collected. Gel retention assays on equivalent samples of protein from the column input, flow-through, step or without protein were as described under Materials and Methods with the amylase minimal enhancer sequence. Arrows denote the position of the free and major shifted bands.

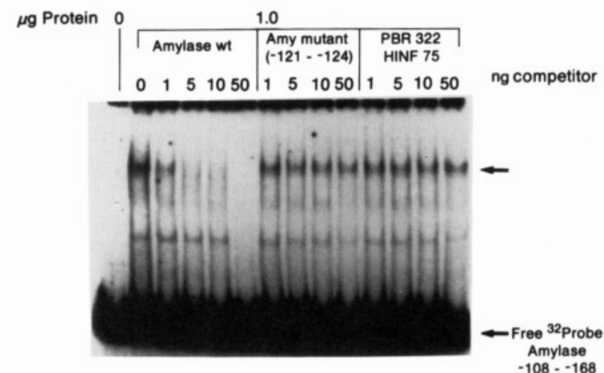

FIGURE 4: Competition analysis of the amylase enhancer binding protein. Approximately 1 µg of the AR42J 0.3 M KCl cellulose phosphate step fraction (Figure 3) was combined with the labeled amylase enhancer diluted with the indicated amounts of nonlabeled competitor DNA fragments. Samples were electrophoresed on a 6% polyacrylamide gel. Arrows indicate the position of the free and major retarded probe.

released at 0.3 M KCl. Other minor and faster migrating bands were also detected, but their presence was variable and nonspecific. No other activities were detected in fractions obtained at higher salt concentrations.

The specificity of the partially purified binding factor(s) was determined by competition assays. As shown in Figure 4, addition of a slight excess of the unlabeled enhancer displaces the binding activity. In contrast, a similar size DNA fragment from pBR322 has essentially no effect, even at high concentrations (Figure 4).

To determine the affinity of the enhancer binding activity, binding of the wild-type sequence was competed with increasing amounts of the amylase mutant IV. As shown in Figure 4, even a 50-fold molar excess of the mutated sequence failed to displace the factor. This result, together with the in vitro footprinting data described above (Figure 2), suggests that the amylase mutation IV alters the DNA binding contacts and greatly lowers the affinity of a binding factor.

The enhancer sequence bound to the partially purified exocrine cell factor(s) was determined by a footprint of the gel-shifted probe. As shown in Figure 5, the enhancer was

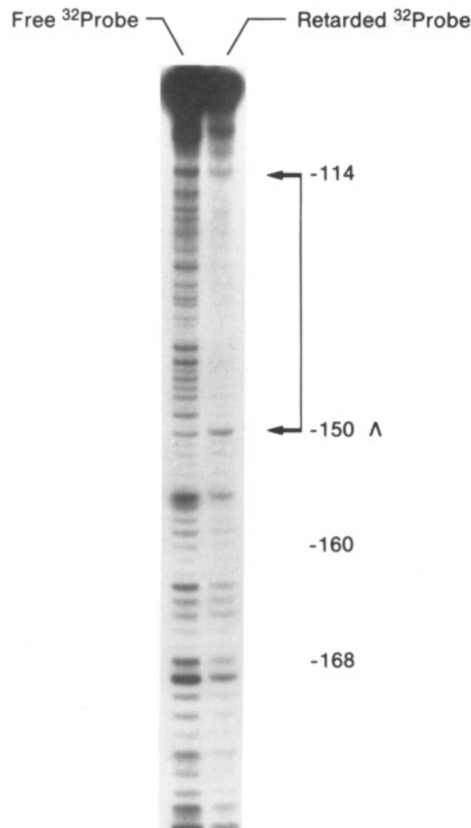

FIGURE 5: DNase I footprint of the amylase enhancer binding protein. A standard binding assay with the 5'-end-labeled amylase enhancer (Figure 2) and the 0.3 M cellulose phosphate fraction of the AR42J cell extract chromatography (see Materials and Methods) was scaled 10-fold and prior to electrophoresis digested with DNase I and resolved on a preparative 7%, low ionic strength polyacrylamide gel. The retarded band was extracted and reelectrophoresed on a 15% 8 M urea sequencing gel. A similarly treated probe in the absence of protein was isolated in parallel (free probe). Numbers are in base pairs from the amylase mRNA CAP site.

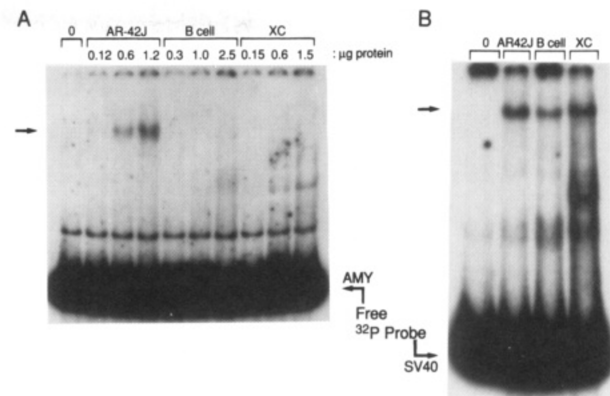

FIGURE 6: Comparison of the amylase enhancer and SV40 72 bp enhancer binding protein in heterologous cell extracts. (A) Whole cell extracts from AR42J cells, mouse lymphoid B cells, and rat XC (fibroblast) cells were prepared and fractionated on cellulose phosphate as described in Figure 5. Gel shift assays were performed with the indicated amount of protein from the 0.3 M KCl fraction as described in Figure 3. (B) One microgram of protein from the 0.3 M KCl cellulose phosphate fraction from AR42J, XC, and B cells was combined with the labeled (*Sph*I–*Pvu*II fragment) enhancer and analyzed as above. The arrows denote the position of the major retarded bands and the free SV40 72 bp enhancer probe.

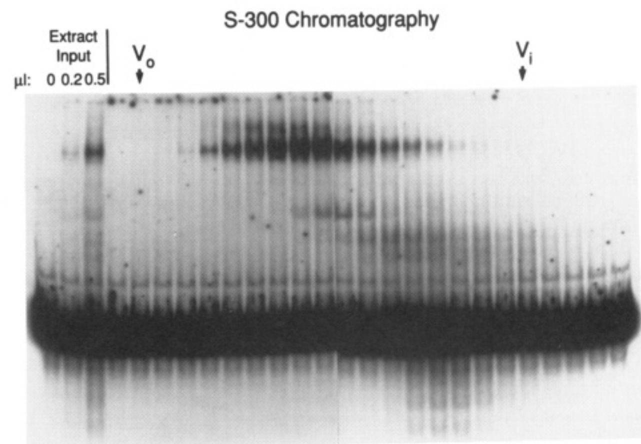

FIGURE 7: Gel filtration of the amylase enhancer binding activity solubilized from canine pancreatic nuclei. The ^{32}P-labeled amylase enhancer was bound to 0, 0.2, or 0.5 μL of pancreatic nuclear extract (7 mg/mL) and analyzed on a 4% low ionic strength gel; 300 μL of the extract was fractionated on a 1.5 × 90 cm column of Sephadex S-300 at a flow rate of 0.5 mL/min in buffer A containing 0.1 M KCl. Five microliters of each fraction was assayed for enhancer binding activity.

protected on the coding strand from −110 to −150.

To assess cell specificity, we compared the enhancer binding activity of the 0.3 M phosphocellulose fraction (see above) from AR42J cells, rat fibroblast (XC) cells, and lymphoid B cells. By electrophoretic mobility shift assays, fractions from AR42J cells contained at least 25-fold higher levels of binding activity (Figure 6a). As shown in Figure 6b, similar levels of SV40 viral enhancer binding proteins were detected in all three extracts. These results suggest that a recognition element of the amylase enhancer interacts with a component(s) enriched in pancreatic exocrine cells and is largely absent or inactive in other cell types under these assay conditions.

The AR42J cell enhancer binding activity is present in the normal differentiated pancreas. Extracts from pancreatic nuclei were prepared and fractionated by Sepharose S-300 gel filtration. A binding assay of the column fractions is shown in Figure 7. Significant levels of binding activity were detected, and by comparison to gel filtration standards possess an estimated mass of 200 kDa under these conditions. This activity was displaced by excess wild-type sequence but not by the amylase mutant IV (data not shown).

The Amylase Enhancer Contains a High-Affinity Binding Site for the SV40 Transcription Factor AP4. The amylase enhancer contains a region, −128CAGCTGTG−121, identical with the factor AP4 binding site in the SV40 late promoter. As expected, affinity-purified AP4 protects both strands of the enhancer (−115 to −136) at concentrations equivalent to those required to saturate the SV40 AP4 site (Mermod et al., 1988), implying that the amylase and SV40 enhancer exhibit approximately the same affinity for AP4. As shown in Figure 8, low levels of AP4 produce a striking DNase I enhancement in the enhancer extending from −140 to −135 on the coding strand and from −112 to −120 on the noncoding strand. Footprint assays with AP4 on the (+30 to −350) amylase promoter–enhancer fragment revealed only the single AP4 binding site at −115 to −136 (data not shown).

The amylase AP4 site is in the 3' domain of the enhancer but represents only a portion of the region protected by the exocrine cell extract factor (Figure 5). As shown in Figure 8, Amy IV mutation–124 to −121 abolishes binding of purified AP4 in vitro. Thus, AP4 and the exocrine factor appear to have common or overlapping DNA binding specificities.

As shown in Table I, the pancreatic consensus sequence differs from the AP4 binding site (Mermod et al., 1988) by only a single G to C transversion. We tested whether the elastase I regulatory region which contains a perfect pancreatic consensus element at −96 to −114 (Boulet et al., 1986; Kruse

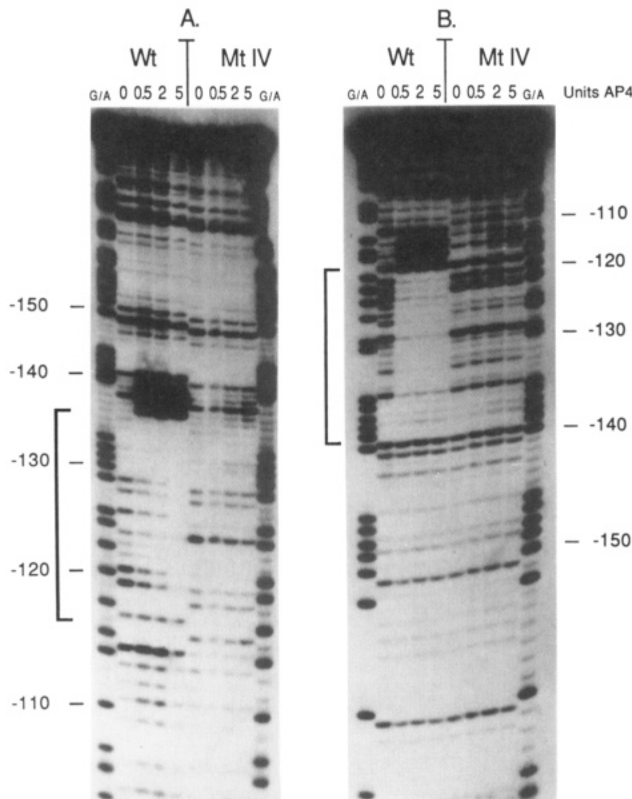

FIGURE 8: Binding of purified AP4 to amylase wild type and Amy mutant IV (−121, −124). The amylase wild-type enhancer or amylase mutant IV was labeled 5′ on the coding (A) or noncoding (B) strand and 1 ng bound to purified AP4 as described (Mermod et al., 1988). Numbers are in base pairs from the amylase CAP site. Brackets indicate the boundaries of protection. One unit corresponds to the amount of protein required to saturate the SV40 AP4 binding site (Mermod et al., 1988).

Table I[a]

Sequence	
CGAGTTTATCCATGAGAGTTTCTGAAGAACCT**TCAGCTGTG**CACATCAATACT	AMY 2A enhancer -161-109
CAGT	AMY mutation IV -124-121
AC**CAGCTGTG**GAA	SV40 AP4 element
CC**CAGCTG**GCG	Elastase AP4 site 1 -65-75
GTCACCTGTGCTTTTCCCTG	Elastase AP4 site 3 -95-114
GTCACCTGTGCTTTTCCCTG	Pancreatic consensus sequence

[a] Horizontal brackets denote the regions of DNase I protection by the exocrine cell factor. The dashed line shows the boundaries of DNase I protection by AP4. Homologous sequences in other regulatory regions are shown in boldface. The Ig heptamer homology is underlined.

et al., 1988) binds AP4. As shown in Figure 9, AP4 binds at −104 to −114 on the coding strand. In addition, two other binding sites for AP4 are detected at −62 to −76 and −85 to −93. Whereas the AP4 core binding sequence is found within the protected region centered at −70, the binding site −93CTTCTACCC−85 does not resemble the canonical AP4 binding site. An additional AP4 binding site has also been noted in the SV40 early region (Mermod et al., 1988), suggesting that alternative sequence elements may be recognized by AP4 (or that undetected DNA binding impurities exist in the AP4 preparation).

Mermod et al. (1988) have noted that other regulatory sequences containing the AP4 binding site also contain binding sites for AP1. Thus, AP1 and AP4 binding sites may act in combination. We therefore carried out DNase I footprint assays with the +30 to −350 amylase 2A promoter–enhancer and affinity-purified AP1 (Lee et al., 1987). Three binding sites for AP1 were found: a high-affinity site at

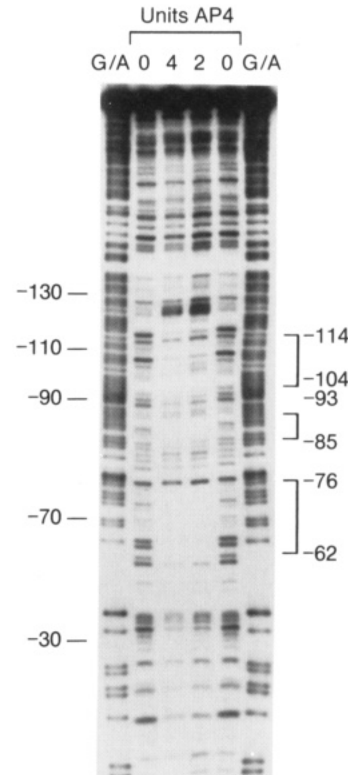

FIGURE 9: Footprint of purified AP4 on pancreatic elastase transcriptional regulatory region. The pancreatic elastase I gene (+8 to −205) (Kruse et al., 1988) was 5′-labeled on the noncoding strand, bound to the indicated amount of AP4 as described in Figure 8, and digested with DNase I. Numbers are in nucleotides from the elastase mRNA CAP site. Brackets denote the major areas of AP4 protection.

−68TGACTA−73 and two others at −114TGATGT−119 and −196TGAGTG−201 (data not shown). Further studies will be required to determine which components of the complex AP1 family bind these motifs, and what influence AP4 may have upon their binding and amylase promoter function in vivo.

Discussion

This report shows that the amylase 2A enhancer is a target for DNA binding factors that are enriched in a pancreatic acinar cell line and in the nuclei of dog pancreatic cells which selectively express this gene. Enhancer domains that are protected from DNase I by the factor(s) are depicted in Table I. On the coding strand, two binding regions (−110 to −130 and −138 to −158) are detected, while on the noncoding strand, a single region (−110 to −160) is observed. A sequence related to the pancreatic consensus sequence is found within this protected region (Table I) and is conserved in the enhancers of pancreatic cell-specific genes (Boulet et al., 1986). As shown in Table I, the recognition site for HeLa transcription factor AP4, 5′-T/CCAGCTGTC/G-3′, also occurs within the consensus element. Amylase mutation IV (Table I) alters the consensus sequence and the AP4 motif and inactivates the enhancer activity in vivo. As shown in this paper, mutation IV abolishes the binding of purified AP4, but does not eliminate the binding of other exocrine cell factors. Presumably these changes lead to a nonproductive enhancer in vivo.

AP4 binding sites have been identified in the proenkephalin, polyoma, SV40, and metallothionein enhancers. In each case, a binding site for the trans-activator AP1 is also present within two helical turns (Mermod et al., 1988). Although the precise action of these trans-activators is unknown, in SV40 AP4 and AP1 participate in driving late viral gene expression from a noncanonical promoter in the A domain of the enhancer

(Mermod et al., 1988; Dynan, 1987). It is likely that the AP1 and AP4 trans-activators may cooperate to regulate transcription in the other enhancers as well. Our footprinting studies with AP1 indicate there are several binding sites within the vicinity of the amylase AP4 element. One of these (−114 to −119) is continuous with the AP4 element (Table I). Two others exist about 60 bp downstream and 70 bases upstream.

The pancreatic consensus AP4 motif exists within a large (50 bp) protected domain and appears to be one element of a complex DNA binding target. We and others (Meister et al., 1989; Roux et al., 1989) have proposed a bipartite or multipartite target site for the pancreatic exocrine cell enhancers. The exocrine cell protein contacts on the chymotrypsin enhancer have been defined (Meister et al., 1989) and a bipartite binding profile over 28 base pairs identified (−213 to −186). Multimeric copies of this sequence are sufficient to elicit acinar cell-specific expression from a minimal promoter element (prolactin −36 to +34), suggesting that this element comprises an essential component of a positive-acting cell-specific determinant.

As we have shown, the 3′ domain of the amylase enhancer contains the AP4 CAGCTG motif. Cockell et al. (1989) have shown that this region binds a 48-kDa protein, similar to the reported size of AP4 (Mermod et al., 1988); however, it is unknown whether the protein detected by Cockell et al. is in fact AP4. Recently our laboratory has cloned two related cDNAs (Pan-1 and -2) encoding proteins that also bind to the CAGCTG motif (Nelson et al., 1990). These proteins have a relative molecular size of 68K and have sequences distinct from AP4 (B. Luscher, personal communication). About 20 base pairs 5′ to the CAGCTG motif is an 8 base pair sequence, TCCCATGG, conserved in pancreatic enhancers which Roux et al. (1989) have shown binds a 64-kDa protein. As shown in Table I, this cognate rat sequence is ATCCATGA. These sequences are similar to the immunoglobulin heptamer sequence CCCATGA that is recognized by Oct 1 and Oct 2 (LeBowitz et al., 1989; Poellinger et al., 1989). The molecular weight of Oct 1 is approximately 100K, and the molecular weight of Oct 2 is approximately 65K. It is not known whether Oct 1 and/or Oct 2 exist in pancreatic cells, and particularly whether protein detected by Roux et al. is related to Oct 2.

Using a 63 base pair fragment of the Amy 2.2 gene enhancer (−110 to −172), Howard et al. (1989) have detected by photo-cross-linking a single binding species of 75 kDa which the authors claim binds in the region −122 to −156. The relationship of this protein complex to the other binding species in the amylase 2A gene is unknown.

Since the transcription of the amylase gene can be regulated by insulin (Korc et al., 1981; Logsdon et al., 1985) and glucocorticoids (Dranginis et al., 1984), it is likely that these hormones influence directly or indirectly the activity of the regulatory region. Presumably the glucocorticoid effect is mediated by its receptor. A sequence with homology to the glucocorticoid receptor binding site has been noted at −134 (Dranginis et al., 1984), which is within the boundary of the exocrine factor(s) reported here. However, direct binding has not been reported. Although a direct action of insulin is not ruled out (insulin is found in the nucleus; Soler et al., 1989), it is attractive that insulin exerts its effects via its receptor, presumably indirectly. Insulin can rapidly increase c-fos transcription in many cells (Rosen, 1987). c-fos dimerizes with the transcription factor c-jun in vivo and may regulate (positively or negatively) genes containing AP1 recognition elements (Halazonetis et al., 1988; Kouzarides & Ziff, 1988). Since three AP1 binding elements exist in the amylase control region, it seems possible that insulin could activate amylase transcription through increases in the c-fos/c-jun complex. This mechanism of insulin induction of amylase activity, however, is not likely to be direct, since insulin exerts its effects over several days.

It is generally believed that gene transcription is regulated by the interaction between trans-acting factors with enhancer and promoter elements in the DNA. It is becoming quite common that different trans-active factors can bind the same or a related DNA element. For example four proteins are identified which bind to CCAAT sequences (Graves et al., 1986; Jones et al., 1985), at least two binding the octamer ATGCAAAT (Mermod et al., 1988; Montminy & Bilezikjian, 1987), and four distinct proteins bind to the AP1 element TGACTC (Lee et al., 1987; Montminy & Bilezikjian, 1987; Bohman et al., 1987). It appears that the CAGCTG motif is also bound by several distinct proteins (Pan-1 and -2 and AP-4). Perhaps only one species may be active in the context of a given enhancer structure, while the others may display negative or neutral properties. Thus, specific gene transcription may be derived not only from the specific array of binding elements in the enhancer but also by the repertoire of specific trans-activators from the group of proteins that can bind a particular element. The active complex might also serve as a target for additional linking proteins which bind the complex and not directly the DNA. Whether the specificity is achieved by combinatorial mechanisms involving nonspecific proteins or if cell-specific proteins are involved at some crucial point in the structure has yet to be ascertained.

Acknowledgments

We thank C. Keleher for contributions to the initial stages of this work and Drs. C. Nelson, D. Melloul, and M. Saenz-Robles for advice and critical reading of the manuscript and Leslie Spector for preparation of the manuscript. We are indebted to Dr. R. Tjian for his generous gift of purified AP4 and AP1.

References

Bohman, D., Bos, T. J., Admon, A., Nishiimurg, T., Vogt, P. K., & Tjian, R. (1987) *Science 238*, 1386–1392.

Boulet, A. M., Erwin, C. R., & Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3599–3603.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Carthew, R. W., Chodosh, L. A., & Sharp, P. A. (1985) *Cell 43*, 437–448.

Cockell, M., Stevenson, B. J., Strubin, M., Hagenbuchle, O., & Wellauer, P. K. (1989) *Mol. Cell. Biol. 9*, 2464–2476.

Dranginis, A., Morley, M., Nesbitt, M., Rosenblum, B., & Meisler, M. (1984) *J. Biol. Chem. 259*, 12216–12219.

Dynan, W. S., & Tjian, R. (1983) *Cell 35*, 79–87.

Dynan, W. S., & Tjian, R. (1985) *Nature 316*, 714–779.

Dynan, W. S. (1987) *Viral Carcinogenesis*, Alfred Benzon Symposium, Munksgaard, Copenhagen.

Fodor, E. J. B., Yen, T. S. B., & Rutter, W. J. (1988) *J. Biol. Chem. 263*, 9569–9572.

Graves, B. J., Johnson, P. F., & McKnight, S. L. (1986) *Cell 44*, 565–576.

Halazonetis, T. D., Gorgopoulos, K., Greenburg, M. E., & Leder, P. (1988) *Cell 55*, 917–924.

Hammer, R. E., Swift, G. H., Ornitz, D. M., Quaife, C. J., Palmiter, R. D., Brinster, R. L., & MacDonald, R. J. (1987) *Mol. Cell. Biol. 7*, 2956–2967.

Howard, G., Keller, P., Johnson, T., & Meisler, M. (1989) *Nucleic Acids Res. 17*, 8185–8195.

Jessop, N. W., & Hay, R. J. (1980) *In Vitro 16*, 212.

Jones, K. A., Yamamoto, K. R., & Tjian, R. (1985) *Cell 42*, 559-572.
Korc, M., Owerbach, D., Quinto, C., & Rutter, W. J. (1981) *Science 213*, 351-353.
Kouzarides, T., & Ziff, E. (1988) *Nature 336*, 646-651.
Kruse, F., Komro, C. T., Michuoff, C. H., & MacDonald, R. J. (1988) *Mol. Cell. Biol. 8*, 893-902.
LeBowitz, J. H., Clerc, R. G., Benowitz, M., & Sharp, P. A. (1989) *Genes Dev. 3*, 1625-1638.
Lee, W., Mitchell, P., & Tjian, R. (1987) *Cell 49*, 741-752.
Logsdon, C. D., Moessner, J., Williams, J., & Goldfine, I. (1985) *J. Cell. Biol. 100*, 1200-1208.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Maniatis, T., Goodburn, S., & Fischer, J. A. (1987) *Science 236*, 1237-1245.
Manley, J. L., Fire, A., Cano, A., Sharp, P. A., & Gefter, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3855-3859.
Maxam, A., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499-525.
Meister, A., Weinrich, S. L., Nelson, C., & Rutter, W. J. (1989) *J. Biol. Chem. 264*, 20744-20751.
Mermod, N., Williams, T. J., & Tjian, R. (1988) *Nature 332*, 557-561.
Montminy, M. R., & Bilezikjian, L. M. (1987) *Nature 328*, 175-180.
Nelson, C., Shen, L., Meister, A., Fodor, E., & Rutter, W. J. (1990) *Genes Dev. 4*, 1035-1043.
Nir, U., Fodor, E., & Rutter, W. J. (1988) *Mol. Cell. Biol. 8*, 982-987.
Ornitz, D. M., Palmiter, R. D., Hammer, R. E., Brinster, R. L., Swift, G. H., & MacDonald, R. J. (1985) *Nature 313*, 600-602.
Osborn, L., Rosenberg, M. P., Keller, S. A., Ting, C.-W., & Meisler, M. (1988) *J. Biol. Chem. 263*, 16519-16522.
Poellinger, L., Yoza, B. K., & Roeder, R. G. (1989) *Nature 337*, 573-576.
Ptashne, M. (1988) *Nature 335*, 683-687.
Rosen, O. (1987) *Science 237*, 1452-1458.
Roux, E., Strubin, M., Hagenbuchle, O., & Wellauer, P. (1989) *Genes Dev. 3*, 1613-1624.
Soler, A. P., Thompson, K. A., Smith, R. M., & Jarett, L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6640-6644.
Walker, M. D., Edlund, T., Boulet, A. M., & Rutter, W. J. (1983) *Nature 306*, 557-561.

Isolation and Amino Acid Sequence Analysis of Bovine Adrenal 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase†

Kay J. Rutherfurd, Shiuan Chen, and John E. Shively*
Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010
Received October 18, 1990; Revised Manuscript Received March 27, 1991

ABSTRACT: 3β-Hydroxysteroid dehydrogenase/steroid isomerase has been purified to homogeneity from bovine adrenal glands. A single protein of molecular weight 42 090 ± 40 containing both enzyme activities has been isolated. Approximately 86% of the amino acid sequence of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase has been obtained by sequencing peptides isolated from digests with trypsin and lysyl endopeptidase and by chemical cleavage with CNBr. The sequence obtained is identical with that of the deduced amino acid sequence of the bovine ovarian 3β-hydroxysteroid dehydrogenase/steroid isomerase [Zhao et al. (1989) *FEBS Lett. 259*, 153-157], with the exception that the N-terminal methionine residue found in the bovine ovarian sequence is not present in the mature bovine adrenal enzyme. On the basis of the primary structure and comparisons with other NAD^+ binding proteins, we propose a structural model of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase localizing the NAD^+ binding site as well as the membrane-anchoring segment.

3β-Hydroxysteroid dehydrogenase and steroid isomerase catalyze consecutive steps in the steroid hormone biosynthesis pathway, namely, the conversion of pregnenolone to progesterone. The enzyme activities of 3β-hydroxy-5-ene steroid dehydrogenase and 5-ene-4-ene steroid isomerase are thought to be catalyzed by a single protein in mammalian tissues (Ford & Engel, 1974; Ishii-Ohba et al., 1986a,b, 1987; Lorence et al., 1990). In contrast, two distinct proteins catalyzing these reactions can be isolated from bacterial sources (Talalay & Wang, 1955; Batzold et al., 1976). The bifunctionality of the mammalian protein has led to interest in the nature of the active site, whether the same steroid binding site may be utilized for both enzyme activities or whether two distinct steroid binding sites exist. Recent reports tend to favor there being two distinct steroid binding sites (Blomquist et al., 1982; Thomas et al., 1990). The critical placement of this enzyme complex at the branch point of the pathway means that 3β-hydroxysteroid dehydrogenase/steroid isomerase plays a crucial role in the biosynthesis of all classes of steroid hormones. Congenital deficiencies or defects at this point in the pathway affect the biosynthesis of estrogens, androgens, and corticosteroids and consequently have far-reaching implications in disorders of steroid metabolism.

Because of the central importance of this enzyme in steroidogenesis, we undertook the complete purification and sequence analysis of the bovine adrenal enzyme. While this work was in progress the complementary DNAs (cDNAs) coding for the human placental and bovine ovarian 3β-hydroxysteroid dehydrogenase/steroid isomerase were cloned, and the amino

†This work was supported in part by research grants from the National Institutes of Health (GM 37297 and HD 14900). The Jasco J-600 spectropolarimeter used in this study was purchased with a National Science Foundation grant (DIR-8804189).

0006-2960/91/0430-8108$02.50/0 © 1991 American Chemical Society