BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **246,** 50–54 (1998)
ARTICLE NO. RC988569

Novel CRE-Binding Proteins of 11-16 kDa Bind to the LDH A-Gene CRE in a Sequence Specific and Hepatocyte-Growth Dependent Manner in Partially Hepatectomized Rat Liver

Mi Young Lee, Eun Sook Hwang, and Seung Ki Lee[1]
College of Pharmacy, Seoul National University, Seoul 151-742, Korea

Received March 29, 1998

We examined cAMP response element (CRE)-binding proteins involved in lactate dehydrogenase A (LDH A)-gene transcription in rat liver after partial hepatectomy. Gel retardation and Southwestern blot assays showed that the CRE-binding activity of the 11-16 kDa novel proteins increased in accordance with increases in LDH A-mRNA in regenerating liver tissues, whereas that of the 43 kDa CREB did not. Using CRE-oligonucleotide affinity chromatography and reverse-phase HPLC, we purified four CRE-binding proteins of 11.2, 15.2, 15.8, and 16.3 kDa. N-terminal amino acid sequences of 15.2 and 16.3 kDa proteins revealed a high sequence homology to but were not identical with those of rat histone H2A.1 and H2B, respectively. CRE-bindings of these two proteins were highly specific, while those of histones H2A.1 and H2B were nonspecific as shown by competition-Southwestern blot and DNase I footprinting assays. Taking these data together, we suggest that the novel 11-16 kDa CRE-binding proteins are responsible for the cell growth-dependent inducibility of LDH A-gene transcription during liver regeneration.

Expression of the cAMP-inducible LDH A-gene has been shown to be regulated at the level of transcription (1, 2). The cAMP response element (CRE) in the promoter region of the mouse LDH A-gene is shown to confer the cAMP inducibility of the gene (3–5).

The 43 kDa CREB (CRE-binding protein) was firstly cloned and identified (6–8). Subsequently, many other types of CREB were identified and grouped to the CREB/ATF family (9). This family belongs to the basic region/leucine zipper (bzip) transcription factor class (10–12). CRE-binding proteins are highly homologous in their bzip region, whereas they are diverse in other parts of the protein. The CRE-binding proteins are functionally divided into activators, CREB, CREM τ (13) and ATF-1 (14, 15), and repressors, CREM α, -β, -γ, and ICER (16). They form homo- or heterodimers by interacting through the leucine zipper regions and thereby acquire different DNA binding specificity and activity.

The cytosolic levels of cAMP have been shown to increase in a triphasic manner in liver tissues after partial hepatectomy (PH), whose second surge of cAMP is functionally linked to the initiation of DNA synthesis (17, 18). We have previously shown that levels of LDH A-mRNA increase in a biphasic manner with peak induction values at 15 and 21 h in liver tissues after PH. The increases were not only temporally related to the intracellular cAMP surges, but also blocked by injecting *dl*-propranolol, a β-adrenergic blocker (19, 20). Thus, we suggested that expression of the LDH A gene is regulated by the cAMP-mediated mechanism in the regenerating rat liver.

In the present report, we examined the CRE-binding proteins involved in the cAMP-inducible LDH A-gene transcription in regenerating liver tissues. The results showed that the LDH A-CRE-binding activity of liver nuclear proteins is mainly attributed to novel 11-16 kDa proteins rather than to the 43 kDa CREB during liver regeneration. N-terminal amino acid sequences of 15.2 kDa and 16.3 kDa proteins were highly homologous but not identical with those of histone H2A.1 and H2B.

MATERIALS AND METHODS

Partial hepatectomy. Liver regeneration was induced by PH in Sprague-Dawley rats, as described by Higgins and Anderson (21).

Preparation of nuclear proteins. Nuclei were prepared from normal and regenerating liver tissues as described by Tsai *et al.* (22) and resuspended in 4 volumes of the lysis buffer (50 mM Hepes, pH 7.4, 0.1% Triton X-100, 150 mM NaCl, 50 mM sodium β-glycerophosphate, 25 mM NaF, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 1 mM

[1] To whom correspondence should be addressed. Fax: +822-889-0751. E-mail: sklcrs@plaza.snu.ac.kr.

0006-291X/98 $25.00

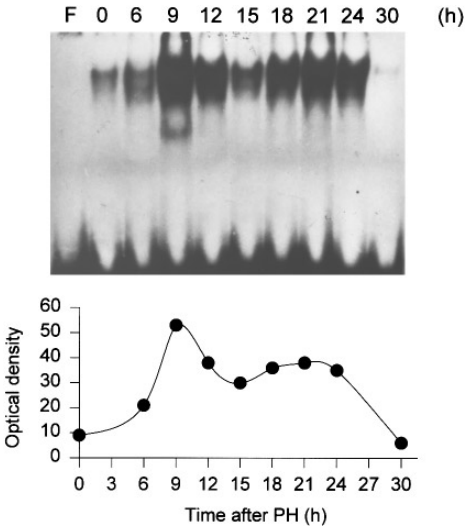

FIG. 1. Temporal changes in the LDH A-CRE binding activity of nuclear proteins during liver regeneration. Nuclear proteins were prepared from liver tissues after PH at the indicated time points and incubated with the LDH A-CRE probe. The CRE-binding activity of nuclear proteins was analyzed by Gel retardation assay and visualized by autoradiography. F indicates the presence of the DNA probe only. The lower panel is the densitometric analysis of upper panel.

PMSF, 1 μg/ml anti-proteolytics). The extracts were used as nuclear proteins.

Gel retardation assay. The gel retardation assay was done as described by SivaRaman *et al.* (23) with a slight modification. The radiolabeled DNA probe was mixed with 1 μg of poly (dI-dC) and 5 μg of nuclear proteins in a binding buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 50 mM NaCl) in a total volume of 25 μl. The DNA sequences used as probes or competitors are LDH A-CRE (5): 5′CCTCACTCTGACGTCAGCGCGCA3′ and somatostatin-CRE (24): 5′GATCTCCTTGGCTGACGTCAGAGAGA3′.

Southwestern blot analysis. Southwestern blot analysis was performed as described by Cowell and Hurst (25): 40 μg of nuclear proteins were resolved by 12 or 18% SDS-PAGE, and electrotransferred onto PVDF membranes. The membranes were blocked for 2 h with TNE-50 buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) containing 5% nonfat milk, and washed twice for 5 min with TNE-50 buffer. Then, the membranes were hybridized with the concatemer LDH A-CRE probe and 20 μg of poly (dI-dC). The concatemer DNA was prepared by 5′-phosphorylation, annealing and ligation of the DNA probe. The concatemer DNA was then radiolabeled by nick translation.

Recovery of the DNA binding proteins from the gel. Recovery of the CRE-binding proteins from SDS-PAGE gel was performed as described by Jackson (26), with a minor modification: 1.2 mg of nuclear proteins were resolved by 12% SDS-PAGE and the gel slices containing 11-16 kDa proteins were homogenized with 10 ml of the elution buffer (150 mM NaCl, 20 mM Hepes, pH 7.5, 5 mM DTT, 0.1 mM EDTA, 0.1 mg/ml BSA, 0.1% SDS). The homogenates were incubated for 1 h at 40°C and filtered through glass filters. The filtrate was precipitated with acetone and the resulting protein precipitate was dissolved in 6 M guanidine-HCl in 0.1 M KCl buffer Z′. After 20 min, the samples were renatured by dialysis against 0.1 M KCl buffer Z′ (25 mM Hepes, pH 7.8, 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 0.1% NP-40, 0.1 M KCl) at 4°C overnight.

DNase I footprinting. DNase I footprinting was carried out as described by Lakin (27). The *Hae* III fragment (74 bp, -94/-20) containing CRE (-48/-41) sequences of the mouse LDH A gene promoter (3) was subcloned into the *Sma* I site of the pWR34 plasmid to generate pWmLC-1. The promoter sequences were PCR-amplified from the pWmLC-1 using 5′-end labeled M13 universal sequencing primers and unlabeled reverse primers and the resulting 203 bp DNA was used as a probe; Protein samples were incubated with 5 ng of the DNA probe and 2 μg of poly (dI-dC) in the binding buffer containing 20 mM Hepes, pH 7.9, 2 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 20% glycerol for 20 min at room temperature. The reaction mixtures were then treated with 50-100 ng of DNase I for 2-3 min at room temperature and 1 volume of the stop buffer (50 mM Tris-HCl, pH 8.0, 2% SDS, 10 mM EDTA, 0.4 mg/ml proteinase K, 100 μg/ml glycogen) was added. The mixtures were further incubated for 30 min at 37°C, extracted with phenol/chloroform, and precipitated with ethanol. The pellets were loaded onto denaturing 6% polyacrylamide gel.

Purification of 11-16 kDa proteins. The sequence-specific DNA affinity column was constructed according to Nicolas and Goodwin (28). LDH A-CRE oligonucleotides were annealed and ligated to produce

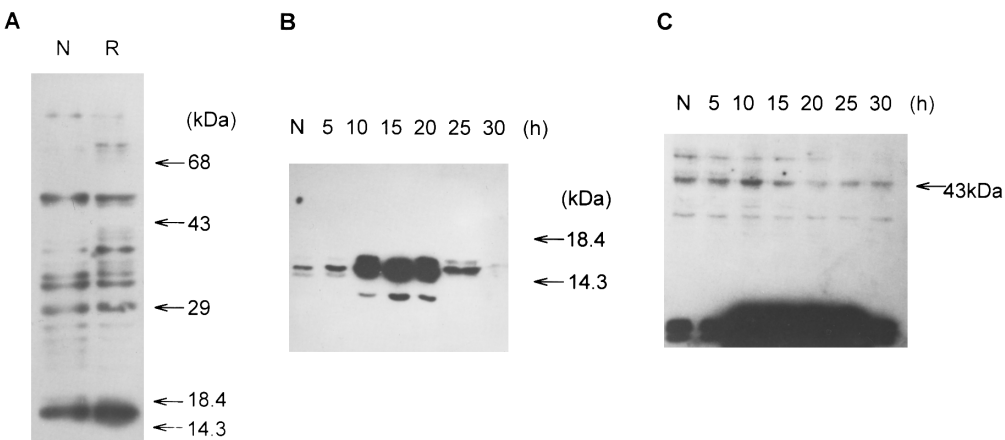

FIG. 2. (A) Southwestern blot analysis of the normal (N) and regenerating (R) liver nuclear extracts. Nuclear proteins were separated by SDS-PAGE and hybridized with LDH A-CRE concatemer as a probe. (B) CRE-binding activity of the low molecular weight CRE-binding proteins after PH. Nuclear proteins prepared at the indicated time points (h) were separated on an 18% SDS-polyacrylamide gel and analyzed by the Southwestern blot method. (C) CRE-binding activity of 43 kDa CREB in liver nuclear proteins extracted at 5-30 h after PH.

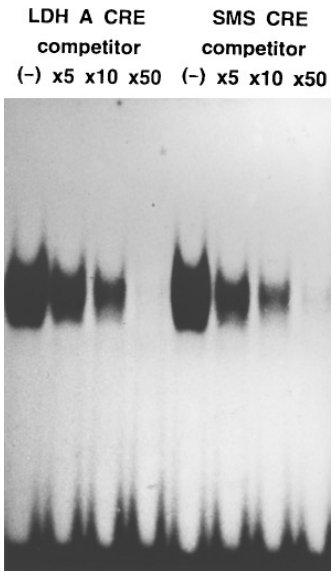

FIG. 3. Competition assay of CRE-binding activity of 11-16 kDa proteins eluted from the gel and renatured as described in Materials and Methods. The gel retardation assay was performed using the indicated fold molar excess of LDH A-CRE and somatostatin-CRE (SMS CRE) sequences as competitors.

concatemer CRE. The concatemer DNA was coupled to CNBr-activated Sepharose 4B. Affinity column chromatography was performed as described by Montminy and Bilezikjian (6). The individual CRE-binding proteins eluted from the affinity column were further purified by reverse-phase HPLC with a Vydac C_4 column. They were separated by a 0 - 85% acetonitrile concentration gradient and the amino acid sequences of the purified proteins were analyzed by microsequencing.

RESULTS AND DISCUSSION

CRE-binding activity of nuclear proteins increases in a biphasic manner during liver regeneration. We examined whether the CRE-binding activity of liver nuclear proteins increases in accordance with the induction of LDH A-gene transcription in regenerating rat liver tissues after partial hepatectomy (PH). Gel mobility shift assays showed that the CRE-binding activity significantly increased with peaking values at 9 and 21 h after PH (Fig. 1). The increases were temporally well related with increases in levels of intracellular cAMP and LDH A-mRNA in liver tissues in that cAMP concentration increases at 2-6 h, 12-14 h and 21 h after PH (17, 18) and levels of LDH A- mRNA increase at 12 and 21 h after PH (19, 20). From the temporal relationships of these molecular events together with our findings, it is suggested that the elevated CRE-binding activity is responsible for the induction of LDH A-gene transcription in regenerating liver tissues.

CRE-binding activity of 11-16 kDa nuclear proteins increases in accordance with increases in levels of LDH A-mRNA in regenerating liver. To examine CRE-binding nuclear proteins involved in the LDH A-gene transcription, we performed Southwestern blot analyses with liver nuclear proteins using LDH A-CRE sequences as a probe. Interestingly, the results showed that the CRE-binding activity of 11-16 kDa proteins strongly increased after PH, while that of the 43 kDa CREB did not (Fig. 2A). Southwestern blottings, after resolving nuclear proteins on an 18% SDS-polyacrylamide gel, showed that the CRE-binding proteins consisted of four different proteins in the 11-16 kDa range, whose CRE-binding activities dramatically increased between 10 and 20 h after PH (Fig. 2B). In contrast, the CRE-binding activity of the 43 kDa CREB was not only shown as a faint band, but also shown as unaltered during the corresponding time period (Fig. 2C). These results suggested that the increased CRE-binding activities of nuclear proteins are mainly attributed to the increased CRE-binding activity of the 11-16 kDa proteins.

11-16 kDa proteins are novel CRE-binding proteins. We examined the possibility that the novel 11-16 kDa CRE-binding proteins are generated from proteolytic degradation of other known CRE-binding proteins in the process of protein extraction. The results from Immunoblot and Southwestern blot assays showed that the CRE-binding activity of 11-16 kDa proteins was even higher in the presence than in the absence of various protease inhibitors, whereas those of the 43 kDa CREB and other CRE-binding proteins were remained unaltered under the same conditions (data not shown). Thus, we concluded that the 11-16 kDa CRE-binding proteins are not the proteolytic products of other CRE-binding proteins with higher molecular weights. We then examined whether the 11-16 kDa CRE-binding proteins bind sequence-specifically to LDH A-CRE. For this, we recovered 11-16 kDa proteins from the SDS-

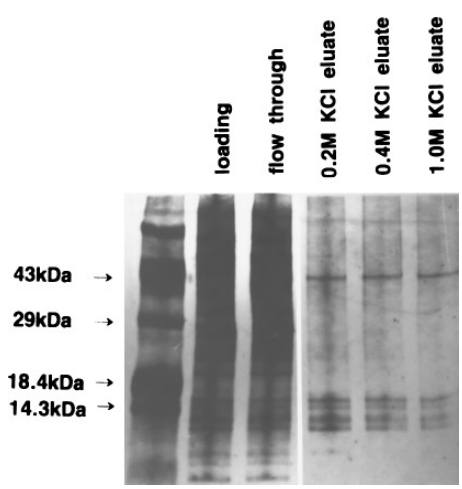

FIG. 4. DNA affinity column chromatography of nuclear proteins. 25 mg of nuclear proteins were loaded on the 1 ml CRE-affinity column and CRE-binding proteins were eluted with buffer Z/1 M KCl. Nuclear proteins in 1 μl of the eluates were resolved on a glycerol-20% polyacrylamide gel and detected by silver staining.

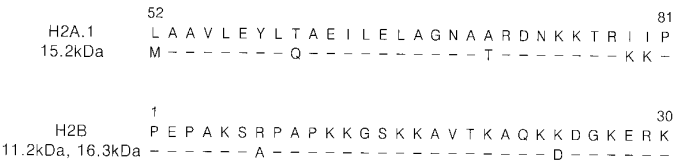

FIG. 5. Comparison of amino acid sequences of 11-16 kDa CRE-binding proteins and H2A.1 and H2B.

polyacrylamide gel and renatured the proteins for gel retardation assay. As shown in Fig. 3, the CRE-binding activity of the 11-16 kDa proteins completely disappeared when competing with a 50-fold higher concentration of specific competitors, LDH A-CRE and somatostatin-CRE sequences. The results clearly suggested that the 11-16 kDa nuclear proteins specifically bind to the CRE sequences.

Purification and analysis of N-terminal amino acid sequences of the 11-16 kDa CRE-binding proteins. To purify the 11-16 kDa CRE-binding proteins, we prepared a DNA affinity column by coupling LDH A-CRE concatemers to CNBr-activated Sepharose 4B. Using DNA affinity chromatography, we were able to efficiently enrich 43 kDa and 11-16 kDa CRE-binding proteins (Fig. 4). We further purified the 11-16 kDa proteins using reverse-phase HPLC with a Vydac C_4 column, and found that the proteins consisted of four different kinds of proteins with apparent molecular masses of 11.2, 15.2, 15.8, and 16.3 kDa. Purified proteins were then cleaved with CNBr and N-terminal amino acid sequences of the products were analyzed by the microsequencing method. The results showed that the amino acid sequences of the 15.2 kDa protein were similar, but not identical to those of histone H2A.1 with 82% of amino acid sequence homology. And the amino acid sequences of the 16.3 kDa protein were similar to those of H2B with a 93% homology (Fig. 5). However, it is well known that the amino acid sequences of histones, H2A and H2B are almost perfectly conserved among species. Thus, it is evident that the 11-16 kDa proteins are different proteins from histones.

CRE-binding specificity of 11-16 kDa nuclear proteins. Since N-terminal amino acid sequences of the 11-16 kDa proteins revealed a high sequence homology to those of rat histones, H2A.1 and H2B, we compared the CRE-binding specificity of the 11-16 kDa proteins with those of H2A and H2B. As shown in Fig. 6A, the CRE binding activity of the 11-16 kDa proteins was neutralized by a 10-fold higher concentration of the

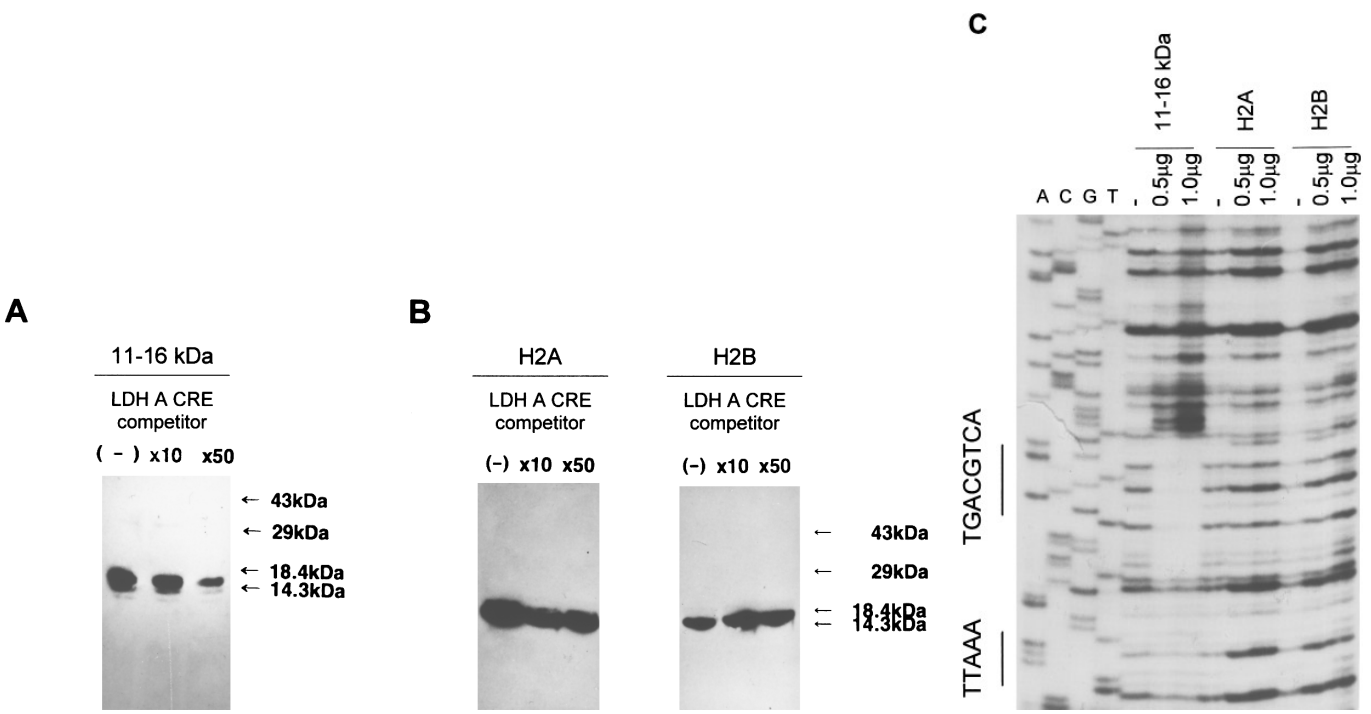

FIG. 6. (A) Competition assay of CRE-binding activity of 11-16 kDa nuclear proteins. 11-16 kDa nuclear proteins were analyzed by the Southwestern blot method in the presence of unlabeled, fold molar excess of LDH A-CRE concatemer as a competitor. (B) Competition assays of CRE-binding activity of H2A and H2B. Histones, H2A and H2B were analyzed by the Southwestern blot method in the presence of unlabeled, fold molar excess of LDH A CRE concatemers as competitors. (C) DNase I footprinting analysis. Mouse LDH A gene promoter fragment was labeled on the sense strand for use as a probe. 11-16 kDa proteins and histones H2A and H2B were incubated with the probe. After treatment with DNase I, the probe was extracted, precipitated, and analyzed. The sequencing results of the LDH A promoter fragment are shown on the left and the CRE (TGACGTCA) sequences and TATA box are indicated.

competitor. In contrast, the CRE binding activities of H2A and H2B were not altered by even a 50-fold higher concentration of the competitor (Fig. 6B). Moreover, the DNase I footprinting assay showed that the LDH A-CRE sequence was protected by binding with 11-16 kDa proteins, but not by binding with H2A or H2B (Fig. 6C). The results suggest that the bindings of H2A and H2B to LDH A-CRE sequences were non-specific, whereas those of the 11-16 kDa proteins were specific interactions.

Taking these data together, we propose that 11-16 kDa histone-related CRE-binding proteins may play an important role in cAMP-inducible LDH A-gene transcription via specific binding to the CRE-sequences during liver regeneration.

ACKNOWLEDGMENTS

This work was supported in part by a Genetic Engineering Research grant from the Ministry of Education and KOSEF through the research center for RCNDD.

REFERENCES

1. Miles, M. F., Hung, P., and Jungmann, R. A. (1981) *J. Biol. Chem.* **256,** 12545–12562.
2. Jungmann, R. A., Kelley, D. C., Miles, M. F., and Milkowski, D. M. (1983) *J. Biol. Chem.* **258,** 5312–5318.
3. Fukasawa, K. M., and Li, S. S.-L. (1986) *Biochem. J.* **235,** 435–439.
4. Fukasawa, K. M., and Li, S. S.-L. (1987) *Genetics* **116,** 99–105.
5. Short, S., Short, M. L., Milkowski, D. M., and Jungmann, R. A. (1991) *J. Biol. Chem.* **266,** 22164–22172.
6. Montminy, M. R., and Bilezikjian, L. M. (1987) *Nature* **328,** 175–178.
7. Gonzalez, G. A., Yamamoto, K. K., Fischer, W. H., Karr, D., Menzel, P., Biggs, W., III, Vale, W. W., and Montminy, M. R. (1989) *Nature* **337,** 749–752.
8. Hoeffler, J. P., Meyer, T. E. Yun, Y., Jameson, J. L., and Habener, J. F. (1988) *Science* **242,** 1430–1433.
9. Lee, K. A. W., and Masson, N. (1993) *Biochimica Biophysica Acta* **1174,** 221–233.
10. Dwarki, V. J., Montminy, M., and Verma, I. M. (1990) *EMBO J.* **9,** 225–232.
11. Yun, Y., Dumoulin, M., and Habener, J. F. (1990) *Molecular Endocrinology* **4,** 931–939.
12. Landschulz, W. H., Johnson, P. F., and Mcknight, S. L. (1988) *Science* **240,** 1759–1764.
13. Foulkes, N. S., Mellstrom, B., Benusiglio, E., and Sassone-Corsi, P. (1992) *Nature* **355,** 80–84.
14. Lee, K. A. W., Hai, T., Sivaraman, L., Thimmappaya, B., Hurst, H., Jones, N. C., and Green, M. R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8355–8359.
15. Hai, T., Liu, F., Coukos, W. J., and Green, M. (1989) *Genes Dev.* **3,** 2083–2090.
16. Molina, C. A., Foulkes, N. S., Lalli, E., and Sassone-Corsi, P. (1993) *Cell* **75,** 875–886.
17. MacManus, J. P., Franks, D. J., Youdale, T., and Braceland, B. M. (1972) *Biochem. Biophys. Res. Commun.* **49,** 1201–1207.
18. MacManus, J. P., Braceland, B. M., Youdale, T., and Whitfield, J. F. (1973) *J. Cell. Physiol.* **82,** 157–164.
19. Kim, H. Y., and Lee, S. K. (1988) *Yakhak Hoeji* **32,** 239–244.
20. Yhu, S. K., Park, H. S., Kim, H. Y., Kim, A. K., and Lee, S. K. (1988) *Arch. Pharm. Res.* **11**(4), 278–284.
21. Higgins, G. M., and Anderson, R. M. (1931) *Arch. Pathol.* **12,** 186–202.
22. Tsai, M.-J., Schwartz, R. J., Tsai, S. Y., and O'Malley, B. W. (1975) *J. Biol. Chem.* **250,** 5165–5174.
23. SivaRaman, L., Subramanian, S., and Thimmappaya, B. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5914–5918.
24. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6682–6686.
25. Cowell, I. G., and Hurst, H. C. (1993) *in* Transcription Factors: A Practical Approach (Latchman, D. S., Ed.), pp. 105–123, Oxford Univ. Press, New York.
26. Jackson, S. P. (1993) *in* Gene Transcription: A Practical Approach (Hames, B. D., and Higgins, S. J., Eds.), pp. 189–242, Oxford Univ. Press, New York.
27. Lakin, N. D. (1993) *in* Transcription Factors: A Practical Approach (Latchman, D. S., Ed.), pp. 27–47, Oxford Univ. Press, New York.
28. Nicolas, R. H., and Goodwin, G. H. (1993) *in* Transcription Factors: A Practical Approach (Latchman, D. S., Ed.), pp. 82–104, Oxford Univ. Press, New York.