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Evidence for nuclear targeting of prothymosin and parathymosin synthesized *in situ*

(nuclear location signal/*in vitro* expression)

M. CLINTON*†, L. GRAEVE*‡, H. EL-DORRY*§, E. RODRIGUEZ-BOULAN¶, AND B. L. HORECKER*‖

Departments of *Biochemistry and ¶Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

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ABSTRACT **To test the hypothesis that prothymosin and parathymosin contain amino acid sequences that cause them to be targeted to the cell nucleus, expression vectors were constructed containing a simian virus 40 promoter and cDNAs that would code for chimeric proteins composed of truncated human growth hormone (hGH) linked to the NH_2 terminus of prothymosin or parathymosin. The truncated hGH lacked the signal peptide sequence required for its secretion. After transfection of these constructs into HeLa S3 cells, which do not normally synthesize hGH, the use of indirect immunofluorescence staining to follow the localization of the hGH chimeras demonstrated that both prothymosin and parathymosin caused targeting to the cell nucleus. Controls with a construct coding for native hGH only, and one coding for the truncated hGH lacking the signal peptide, revealed secretion into culture medium and staining in the endoplasmic reticulum and Golgi apparatus in the first case, and diffuse staining throughout the cytoplasm in the second. The results provide direct evidence, with proteins synthesized *in situ*, for the presence of nuclear localization signals in both prothymosin and parathymosin.**

Interest in proteins of thymic origin was stimulated by the observation that a crude preparation from calf thymus, designated thymosin fraction 5, showed immunomodulatory properties in a number of *in vitro* test systems (reviewed in ref. 1). Many of these properties were attributed to a single peptide purified from this fraction, named thymosin α_1 (2), which was proposed to function as a "thymic hormone" influencing lymphocyte maturation (3). Based on evidence suggesting that this thymosin α_1 might be a proteolytic artifact that arose during the preparation of thymosin fraction 5 (4, 5), a search was initiated for the native protein or polypeptide precursor. A procedure designed to minimize endogenous proteolysis led to the isolation of two homologous polypeptides, named prothymosin (6) and parathymosin (7), the first of which contained the 28-amino acid sequence of thymosin α_1 at its NH_2 terminus. The proposed role of thymosin α_1 as a thymic hormone was brought into question when the putative precursor, prothymosin, and the mRNA coding for this polypeptide were shown to be present in all mammalian tissues examined (8, 9). In addition, the cDNAs coding for both human (10, 11) and rat (12) prothymosins were found to lack sequences coding for the signal peptides expected in secretory proteins. An extracellular function was also rendered unlikely by the observation that the mRNA for prothymosin was localized exclusively on free polysomes (13).

The experimental findings relating to parathymosin have closely mimicked those for prothymosin, with quantitative differences in tissue polypeptide and mRNA content (7, 9, 12, 14).

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More recent studies have suggested a relationship between prothymosin and cell growth. Increased prothymosin mRNA in response to a variety of stimulators of mammalian cell division has been reported (11), and elevated mRNA was also found in lymphocytes from leukemic patients (15). In a rat small intestinal cell line, levels of thymosin-immunoreactive peptide, either thymosin α_1 or prothymosin, were sharply elevated during cell proliferation, particularly during the G_1 phase of the cell cycle (16). Down-regulation of prothymosin mRNA was also observed in an HL-60 myeloid leukemia cell line induced to differentiate with either dimethyl sulfoxide or phorbol 12-myristate 13-acetate (36). In other reports, prothymosin has been shown to be covalently linked to a small cytoplasmic RNA (17), and parathymosin has been identified as the Zn^{2+}-binding protein that reversibly inactivates phosphofructokinase (18).

Recent evidence has also raised the possibility of a nuclear site of action for both prothymosin and parathymosin. On the basis of the presence of a "nuclear localization signal" near the COOH terminus of prothymosin and superficial structural similarities between prothymosin and a variety of nuclear proteins, Gomez-Marquez and Segade (19) proposed that it might function as a nuclear protein. Although a conventional cell-fractionation procedure yielded detectable levels of prothymosin only in the cytoplasmic fraction of calf thymus or liver (20), an elegant series of experiments by Watts *et al.* (21, 22) demonstrated that both prothymosin and parathymosin injected into *Xenopus* oocytes accumulated in the nucleus, in contrast to thymosin β_4, which remained in the cytoplasm. Using an immunogold labeling procedure, Conteas *et al.* (16) also found a nuclear concentration of the thymosin-immunoreactive peptide that they observed to increase on initiation of the cell cycle. Prothymosin has been reported to copurify with high-mobility-group proteins in perchloric acid extracts of rat thymus nuclei (23).

The present study was initiated to clarify the question of nuclear targeting of prothymosin and parathymosin, since a clear demonstration of their intracellular location would be invaluable in the determination of the precise cellular function of these ubiquitous compounds. We report that both polypeptides, synthesized *in situ* from their respective cDNAs, are indeed targeted to the cell nucleus. For this purpose, constructs were designed which coded for prothymosin or parathymosin linked to human growth hormone (hGH), which is not normally present in HeLa S3 cells, as a "reporter molecule."

Abbreviation: hGH, human growth hormone.

†Present address: National Institute of Medical Research, Mill Hill, London NW7 1AA, United Kingdom.
‡Present address: Institut für Biochemie, Rheinisch-Westfälische Technische Hochschule Aachen, D-5100 Aachen, Federal Republic of Germany.
§Present address: Instituto de Quimica, Universidade de Sao Paulo, Sao Paulo, Brazil.
‖To whom reprint requests should be addressed.

MATERIALS AND PROCEDURES

Materials

HeLa S3 cells (American Type Culture Collection, CCL 2.2) were grown in 100-mm plastic dishes in the presence of Dulbecco's minimal essential medium (GIBCO) with 10% fetal calf serum (HyClone). Goat antiserum directed against hGH was a generous gift from Genentech. Rhodamine-conjugated rabbit anti-goat IgG was purchased from Cappel Laboratories. Bluescript KS M13 was purchased from Stratagene. BLpSV was obtained from M. Rosen (Roche Institute for Molecular Biology, Nutley, NJ). pMV-7 (24) was a gift from M. Chao (Cornell University Medical College, New York).

Experimental Procedures

Construction of Expression Vectors. The cDNA for hGH was isolated from a human pituitary library, prepared and screened at the University of Sao Paulo, Brazil. It was subcloned in Bluescript KS M13 and designated GS-GH-8. Prothymosin and parathymosin DNAs were the cDNA clones pHSpro565 (10) and pHK41188 (14), respectively. Specific oligonucleotides (Fig. 1*A*) were used in conjunction with the PCR to insert suitable restriction enzymes sites and amplify the relevant cDNAs to enable subcloning in the expression vector BLpSV, which contains a simian virus 40 promoter and polyadenylylation signal and a multiple cloning site.

Constructs were designed for expression of the following protein products: I, native hGH; II, truncated hGH, lacking the signal peptide necessary for secretion; III, truncated hGH fused to the NH_2 terminus of prothymosin; and IV, truncated hGH fused to the NH_2 terminus of parathymosin (Fig. 1*B*).

Expression in HeLa S3 Cells. For each construct, HeLa S3 cells were cotransfected with expression vector DNA and DNA from pMV-7, a vector conferring resistance to the drug G418. HeLa S3 cells were transfected by the calcium phosphate procedure and selected in medium containing G418 at 400 μg/ml as described (25). Cells were selected over a 10-day period to obtain resistant clones.

Stably transfected cells were initially screened by immunofluorescence; approximately 75% of the clones were found to express growth hormone constructs. For analysis of transfectants, cells were plated on glass coverslips, grown for 2 days, and fixed with 2% (wt/vol) paraformaldehyde. After permeabilization with 0.075% saponin, cells were incubated with goat anti-hGH, stained with rhodamine-conjugated rabbit anti-goat IgG, examined by fluorescence light microscopy with an epifluorescence microscope (Leitz), and photographed with 400 ASA film (Kodak Tri-X) (26).

Extracts from transfected cells were subjected to SDS/PAGE on 12% acrylamide gels, transferred to nitrocellulose, incubated sequentially with goat anti-hGH, rabbit anti-goat IgG, and ^{125}I-labeled staphylococcal protein A, and subjected to autoradiography (27). For analyses of culture media, cells were pulse labeled for 1 hr with Expre^{35}S^{35}S (NEN) in methionine- and cysteine-free medium followed by a chase period of 3 hr in complete medium containing 0.2% bovine serum albumin. The medium was immunoprecipitated with the goat antiserum directed against hGH followed by protein A-Sepharose, and immunoprecipitates were analyzed by SDS/12% PAGE and fluorography (28).

RESULTS

Nuclear Localization of Chimeric Proteins. The specific approach was to prepare expression constructs coding for chimeric proteins composed of hGH fused to either the NH_2 terminus of prothymosin or the NH_2 terminus of parathy-

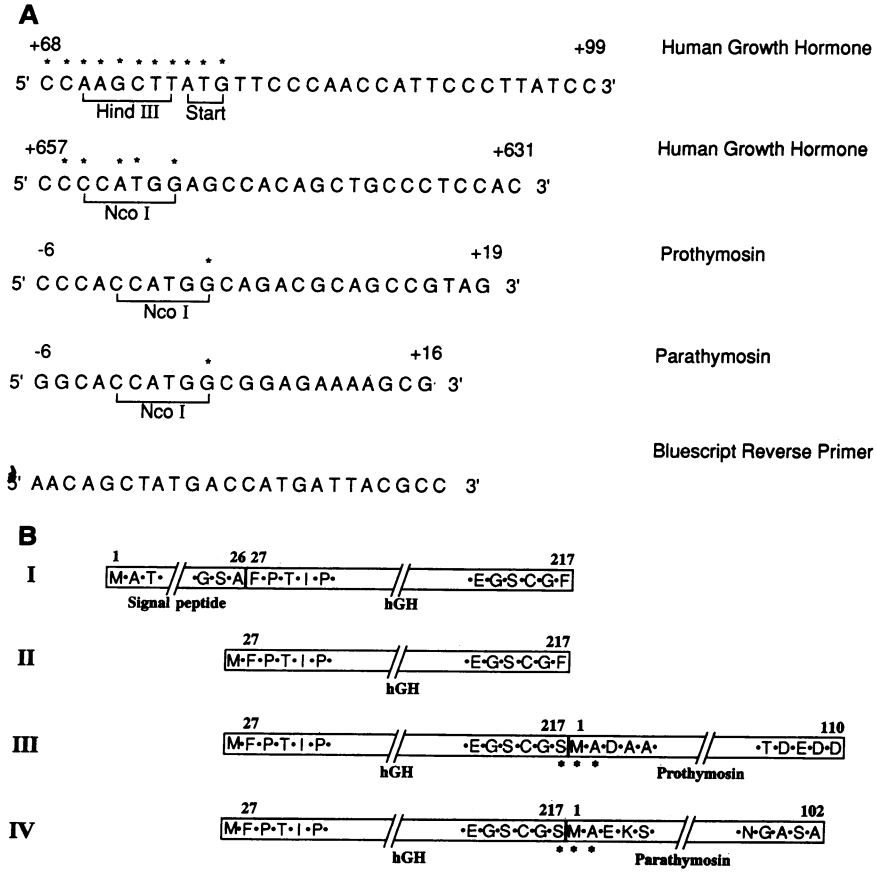

FIG. 1. (*A*) Oligonucleotides used in PCR amplification of cDNAs of mutated forms of hGH. To amplify construct II cDNA, hGH (+68 to +99) oligonucleotide was used in conjunction with the Bluescript reverse primer. To amplify cDNA for the fusion constructs (III and IV), prothymosin or parathymosin oligonucleotide was used in conjunction with the Bluescript primer, while the hGH moiety was prepared with both hGH oligonucleotides. Numbers above sequences refer to corresponding nucleotides in the cDNA sequences, while asterisks denote changes from the cDNA sequences. Engineered restriction enzyme sites and translation start codons are shown below oligonucleotide sequences. (*B*) Protein products of expression constructs coding for hGH and mutated forms. I, native hGH; II, truncated hGH; III, truncated hGH fused to prothymosin; and IV, truncated hGH fused to parathymosin. Numbers refer to corresponding amino acids in the protein sequence, while asterisks denote changes from the protein sequences. Letters identifying amino acids are limited to the NH_2 and COOH termini of individual components.

mosin (Fig. 1*B*). Upon transfection of HeLa S3 cells and establishing stable expressing cell lines, the positional fate of the newly synthesized proteins was determined by indirect immunofluorescence using a polyclonal antibody directed against hGH. The use of hGH, not normally present in HeLa S3 cells, as a "reporter molecule" enabled the specific detection of newly synthesized chimeric proteins without interference from endogenous prothymosin and parathymosin. Analysis of cells transfected with construct I, containing the cDNA for native hGH, revealed that fluorescence was confined to the perinuclear region typical for a location in the endoplasmic reticulum and Golgi compartment, while no fluorescence was observed in the nucleus itself (Fig. 2*A*). In cells transfected with construct II, containing cDNA coding for truncated hGH lacking the signal peptide responsible for secretion, there was again no evidence for localization of fluorescence to the secretory apparatus or the nucleus; rather, diffuse staining was observed throughout the cytoplasm (Fig. 2*B*). In contrast, in cells transfected with DNA coding for the chimeric proteins, truncated hGH fused to either prothymosin or parathymosin, the nucleus stained strongly, with little or no staining evident elsewhere in the cell (Fig. 2 *C* and *D*). The nuclear staining was more prominent with the parathymosin chimera, indicating that higher levels of this protein were expressed (see also Fig. 3*A*). The nuclear staining evident in these cells appeared to be excluded from the nucleolar compartment.

Molecular Size of Expressed Proteins. As prothymosin and parathymosin localization was performed by means of a reporter molecule, which may not necessarily reflect the presence of the intact protein product, the integrity of the expressed polypeptide was examined in transfected cells and culture media. For this purpose, extracts from transfected cells were subjected to SDS/PAGE, transferred to nitrocellulose, and incubated with goat anti-hGH followed by rabbit anti-goat IgG and ^{125}I-protein A (Fig. 3*A*). Analysis of commercially available hGH revealed two major bands, one with the expected molecular mass of 22 kDa and one with the apparent molecular mass of ≈38 kDa, the latter most likely representing hGH dimer (29). Extracts from nontransfected

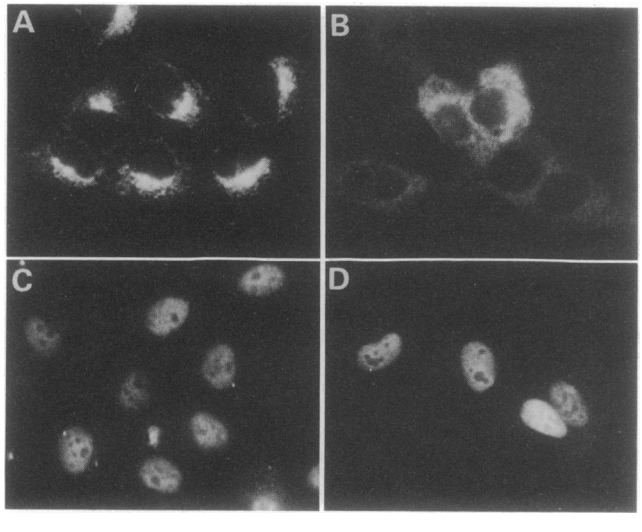

FIG. 2. Indirect immunofluorescence localization of hGH and its mutated forms in transfected HeLa S3 cells. Cells were transfected with constructs coding for native hGH (*A*), truncated hGH lacking the signal sequence (*B*), truncated hGH–prothymosin chimera (*C*), or truncated hGH-parathymosin chimera (*D*). Cells were plated on glass coverslips, grown for 2 days, and fixed. After permeablization, cells were incubated with rabbit anti-hGH, stained with rhodamine-conjugated anti-rabbit IgG, examined by fluorescence microscopy, and photographed. (×280.)

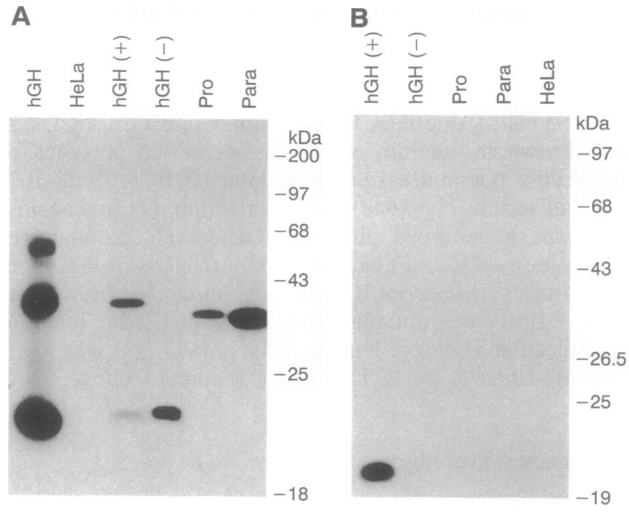

FIG. 3. (*A*) Expression of hGH and its mutated forms in transfected HeLa S3 cells. Cell extracts (1×10^6 cells) of control cells (HeLa) and cells transfected with constructs coding for native hGH [hGH(+)], truncated hGH [hGH(−)], and prothymosin and parathymosin chimeras (Pro, Para) were subjected to SDS/PAGE. After transfer to nitrocellulose, the membrane was incubated with polyclonal goat antiserum directed against hGH (1:5000), followed by rabbit antiserum against goat IgG (1:1000) and ^{125}I-protein A. A similar analysis of commercially available hGH was included in the leftmost lane for comparison. The fluorogram of the blot is shown. Numbers refer to sizes of molecular mass markers. (*B*) Immunoprecipitation of media of transfected cells. Cells (1.5×10^6) were labeled for 3 hr with 100 μCi (3.7 MBq) of Expre^{35}S (NEN) in 500 μl of methionine- and cysteine-free medium. The medium was centrifuged for 15 min at 12,000 × *g* to remove cellular debris, adjusted to 1% Triton X-100, and mixed with 1 μl of goat antiserum directed against hGH followed by 5 μl of rabbit antiserum against goat IgG and protein A-Sepharose. Immunoprecipitates were analyzed by SDS/PAGE. Gels were fixed and fluorographed, using sodium salicylate as enhancer.

HeLa S3 cells contained no proteins that reacted with anti-hGH, while extracts from cells transfected with cDNA coding for native hGH contained two proteins of molecular masses identical to those of the proteins present in commercially available hGH. Analysis of extracts from cells expressing truncated hGH revealed a single immunoreactive band of similar size to monomeric hGH. Extracts from cells expressing the hGH-prothymosin and hGH-parathymosin chimeric proteins each contained a single immunoreactive protein of ≈34 kDa, consistent with the expected size of hGH fused to intact prothymosin or parathymosin.

For analyses of culture media, cells were metabolically labeled with Expre^{35}S in methionine- and cysteine-free medium for 1 hr and chased for an additional 3 hr. The medium was then immunoprecipitated with the goat antiserum directed against hGH, and immunoprecipitates were analyzed by SDS/PAGE and fluorography (Fig. 3*B*). Measurable quantities of immunoreactive material were detected only in medium of cells transfected with the cDNA coding for native hGH, clearly demonstrating that neither truncated hGH nor the chimeric forms are secreted by the cells.

DISCUSSION

These results confirm the validity of the experimental system: native hGH is confined to the secretory apparatus and is detected in the culture medium, while truncated hGH is diffusively distributed in the cytoplasm. Interestingly, although truncated hGH is found throughout the cytoplasm and is of a size reportedly small enough to diffuse through the nuclear pores (30), no staining is observed within the nucleus.

In marked contrast, when truncated hGH is expressed as a chimeric protein fused to either prothymosin or parathymosin, the nucleus stains brightly, providing direct evidence to support the proposal that prothymosin and parathymosin contain nuclear targeting sequences that function when the proteins are synthesized *in situ*. For human prothymosin and parathymosin, the targeting sequences would presumably be Asp-Thr-Lys-Lys-Gln-Lys-Thr (residues 100–106) (10) and Pro-Lys-Arg-Gln-Lys-Thr (residues 90–95) (14), respectively. Although the chimeric proteins are small enough to diffuse into the nucleus and the possibility exists that the accumulation observed within the nucleus is the result of specific retention of diffused proteins, this is unlikely for two reasons. First, a fragment of this protein, truncated hGH, does not diffuse into the nucleus, and second, in such a situation, quantities of newly synthesized chimeric proteins should be detectable diffused throughout the cystoplasm, and this is not the case. It appears more likely that, on synthesis, both prothymosin and parathymosin are specifically directed into the nucleus.

From the literature (16, 17, 20) it seems obvious that prothymosin, at least, is also found in the cytoplasm. This could be the result of specific removal of prothymosin from the nucleus by linkage to a small RNA, as described by Makarova *et al.* (17). In our experimental system, the chimeric nature of the protein may somehow inhibit the return of prothymosin and parathymosin to the cytoplasm.

On the basis of the structure of prothymosin, some workers have suggested that it may be associated with the nucleolus; from this study it would appear to be excluded from the nucleolar compartment. However, this aspect of thymosin behavior may also conceivably be altered by the chimeric structure of the protein.

Despite the lack of a signal sequence, a theory has persisted that prothymosin is a secretory protein. The data presented here argue strongly against this possibility, as neither chimeric protein follows the accepted pathway of secretion as delineated by expression of native hGH. In the event that a specific NH_2-terminal proteolytic fragment of prothymosin—e.g., thymosin α_1—was secreted by an alternative pathway as described recently for fibroblast growth factor and interleukin 1β (31–33), this product would exist in our study as an hGH–thymosin-α_1 chimera and should have been detected in the culture medium. It is conceivable that the chimeric protein structure may interfere with a secretion through this pathway, but this seems a less likely alternative.

The results reported here provide direct evidence for the proposal by Gomez-Marquez and Segade (19) that prothymosin contains a nuclear targeting signal, and they complement and support the data described by Watts *et al.* (21, 22) and Conteas *et al.* (16). They also suggest that, upon synthesis, both prothymosin and parathymosin are specifically directed into the nonnucleolar compartment of the nucleus. Although parathymosin does not contain a "classical" nuclear localization signal, as described by Gomez-Marquez and Segade (19), a possible candidate for such a signal is the region from residue 91 to residue 94, which differs from the identified sequence by the substitution of arginine for lysine at position 92.

Although the precise roles of prothymosin and parathymosin remain unclear, it is interesting to note that a direct link has been established between prothymosin and myc, a protein implicated in DNA replication and regulation of gene expression (reviewed in ref. 34). In a recent study (35), the stimulation of cell division by activation of myc resulted in elevated prothymosin mRNA, independent of protein synthesis, indicating a direct effect of the *MYC* product on prothymosin gene transcription. This result suggests a role for prothymosin in cell proliferation, early in the cascade of events following mitogenic stimulation.

Note Added in Proof. While this manuscript was in preparation, the results of a similar study on one of these proteins were reported by Manrow *et al.* (37). These workers expressed prothymosin/β-galactosidase chimeric proteins in COS cells and reported evidence for nuclear localization based on isolation of sealed nuclei and indirect immunofluorescence. Although they did not report analyses of culture media, these authors have extended our findings by expressing chimeric proteins containing the NH_2-terminal or COOH-terminal regions of prothymosin and have confirmed that the nuclear localization signal lies in the COOH-terminal regions of prothymosin, probably in the sequence Thr-Lys-Lys-Gln-Lys-Thr. They did not report experiments with constructs containing nucleic acid sequences for parathymosin.

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1. Low, T. L. K. & Goldstein, A. L. (1985) *Methods Enzymol.* **116,** 219–247.
2. Goldstein, A., Low, T. L. K., McAdoo, M., McClure, J., Thurman, G. B., Rossio, J., Lai, C.-Y., Chang, D., Wang, S.-S., Harvey, C., Ramel, A. H. & Meienhofer, J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 725–729.
3. Oates, K. K. & Goldstein, A. (1984) *Trends Pharmacol. Sci.* **60,** 347–352.
4. Hannappel, E., Davoust, S. & Horecker, B. L. (1982) *Biochem. Biophys. Res. Commun.* **104,** 266–271.
5. Caldarella, J., Goodall, G. J., Felix, A. M., Heimer, E. P., Salvin, S. B. & Horecker, B. L. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7424–7427.
6. Haritos, A. A., Goodall, G. J. & Horecker, B. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1008–1011.
7. Haritos, A. A., Salvin, S. B., Blacher, R., Stein, S. & Horecker, B. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1050–1053.
8. Haritos, A. A., Tsolas, O. & Horecker, B. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1391–1393.
9. Clinton, M., Frangou-Lazaridis, M., Panneerselvam, C. & Horecker, B. L. (1989) *Arch. Biochem. Biophys.* **269,** 256–263.
10. Goodall, G. J., Dominguez, F. & Horecker, B. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8926–8928.
11. Eschenfeldt, W. H. & Berger, S. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9403–9407.
12. Frangou-Lazaridis, M., Clinton, M., Goodall, G. J. & Horecker, B. L. (1988) *Arch. Biochem. Biophys.* **263,** 305–310.
13. Eschenfeldt, W. H., Manrow, R. E., Krug, M. S. & Berger, S. L. (1989) *J. Biol. Chem.* **264,** 7546–7555.
14. Clinton, M., Frangou-Lazaridis, M., Panneerselvam, C. & Horecker, B. L. (1989) *Biochem. Biophys. Res. Commun.* **158,** 855–862.
15. Gomez-Marquez, J., Segade, F., Dosil, M., Pichel, J. G., Bustelo, X. R. & Freire, M. (1989) *J. Biol. Chem.* **264,** 8451–8454.
16. Conteas, C. N., Mutchnick, M. G., Palmer, K. C., Weller, F. E., Luk, G. D., Naylor, P. H., Erdos, M. R., Goldstein, A. L., Panneerselvam, C. & Horecker, B. L. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3269–3273.
17. Makarova, T., Grebenshikov, N., Egerov, C., Vartapetian, A. & Bogdanov, A. (1989) *FEBS Lett.* **257,** 247–250.
18. Trompeter, H.-I., Brand, I. A. & Soling, H.-D. (1989) *FEBS Lett.* **253,** 63–66.
19. Gomez-Marquez, J. & Segade, F. (1988) *FEBS Lett.* **266,** 217–219.
20. Tsitsiloni, O. E., Yialouris, P. P., Sekeri-Pataryas, K. & Haritos, A. A. (1989) *Experientia* **45,** 332–334.
21. Watts, J. D., Cary, P. D. & Crane-Robinson, C. (1989) *FEBS Lett.* **245,** 17–20.
22. Watts, J. D., Cary, P. D., Sautiere, P. & Crane-Robinson, C. (1990) *Eur. J. Biochem.* **192,** 643–651.
23. Palvimo, J. & Linnala-Kankkunen, A. (1990) *FEBS Lett.* **277,** 257–260.
24. Kirschmeier, P. T., Housey, G. M., Johnson, M. D., Perkins, A. S. & Weinstein, I. B. (1988) *DNA* **7,** 219–225.
25. Rodriguez-Boulan, E., Salas, P. J., Sargiacomo, M., Lisanti,

M., Le Bivic, A., Sambuy, Y., Vega-Salas, D. & Graeve, L. (1989) *Methods Cell Biol.* **32,** 37–55.
26. Graeve, L., Drickamer, K. & Rodriguez-Boulan, E. (1989) *J. Cell Biol.* **109,** 2809–2816.
27. Kessler, S. W. (1975) *J. Immunol.* **115,** 1617–1624.
28. Graeve, L., Patzak, A., Drickamer, K. & Rodriguez-Boulan, E. (1990) *J. Biol. Chem.* **265,** 1216–1224.
29. Bowsher, R. R., Apathy, J. M., Ferguson, A. L., Riggin, R. M. & Henry, D. P. (1990) *Clin. Chem.* **36,** 362–366.
30. Breeuwer, M. & Goldfarb, D. S. (1990) *Cell* **60,** 999–1008.
31. Jaye, M., Howk, R., Burgess, W., Ricca, G. A., Chiu, I.-M., Ravera, M. W., O'Brien, S. J., Modi, W. S., Macaig, T. & Drohan, W. N. (1986) *Science* **233,** 541–545.
32. Lomedico, P. T., Gubler, U., Hellman, C. P., Dukovitch, M., Giri, J. G., Pan, Y.-C. E., Collier, K., Seminow, R., Chua, A. O. & Mizel, S. B. (1984) *Nature (London)* **312,** 458–462.
33. Furutani, Y., Notake, M., Yamayoshi, M., Yamagishi, J., Nomura, H., Ohue, M., Furuta, R., Fukui, T., Yamada, M. & Nakamura, S. (1985) *Nucleic Acids Res.* **13,** 5869–5882.
34. Luscher, B. & Eisenman, R. N. (1991) *Genes Dev.* **4,** 2025–2035.
35. Eilers, M., Schirm, S. & Bishop, M. J. (1991) *EMBO J.* **10,** 133–141.
36. Smith, M. R., Silverman, A., Szabo, P., Kohler, W., Conteas, C. & Mutchnick, M. G. (1990) *Blood* **76,** 246 (abstr.).
37. Manrow, R. C., Shurlatti, A. R., Hanover, J. A. & Berger, S. B. (1991) *J. Biol. Chem.* **266,** 3916–3924.