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Regulation by a protein-free carbohydrate-rich diet of rat pancreatic mRNAs encoding trypsin and elastase isoenzymes

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The levels of mRNAs coding for trypsin and elastase isoenzymic forms were determined in the pancreatic tissue of rats fed a high-carbohydrate protein-free diet for a 0–5-day period. No change in the amounts of mRNAs coding for the two isoelastases was observed, although previous results showed that the biosynthesis of anionic elastase was markedly increased, whereas the biosynthesis of cationic elastase decreased, suggesting the existence of a translational-control mechanism in response to nutritional substrates. In contrast, the levels of mRNAs specific for the three isotrypsins were significantly enhanced, possibly as a result of transcriptional regulation and/or a change in messenger stability. In combination with earlier observations of an overall decrease in cationic trypsin biosynthesis during the same nutritional manipulation, these results suggest that formation of this enzyme is also subject to translational control.

INTRODUCTION

The serine-proteinase family, which accounts for as much as 44% of total protein synthesis in the rat pancreas [1], includes a cationic and two anionic forms of trypsin (EC 3.4.21.4), as well as two chymotrypsins (EC 3.4.21.1) and two elastases (EC 3.4.21.36) with a cationic and an anionic isoenzyme in each case, and several kallikrein-like proteins [2]. All these serine proteinases have comparable M_r values, similar structures and catalytic functions, but quite different substrate specificities [3,4]. Besides the presence of both anionic (I) and cationic (II) elastases in the rat pancreas, a third class of elastase isoenzyme (III) has recently been identified in human pancreatic tissue, by cDNA and genomic gene cloning [5]. However, it is probable that elastase III and one of the human proteinase E isoenzymes are identical, since their cDNA nucleotide sequences are the same [6]. In contrast, Southern-blot analysis [7] has shown that rat pancreatic elastases I and II are encoded by two prominent mRNAs corresponding to distinct specific genes.

The multiple forms of trypsin existing in a number of animal species [8–11] are known to have rather different amino acid sequences and to be specified by distinct genes [12,13]. The nucleotide sequences of the cDNAs encoding cationic trypsin [13] and the two anionic trypsins [14] have been elucidated. However, since the trypsin gene family is composed of at least ten different genes or pseudogenes [12], the number of trypsin isoenzymes in the rat pancreatic tissue should exceed three. This may be of importance with respect to the regulation of gene expression in response to nutritional substrates or hormones.

Although it has been recognized for a long time that the levels of pancreatic hydrolases are regulated by the composition of the diet [15–18], the question as to whether nutritional factors exert their effect on protein synthesis directly at any point in the gene-expression process or, indirectly, through hormonal stimulation, is still open. In a previous study [19] we reported that ingestion of a protein-free carbohydrate-rich diet resulted in a marked increase in the cellular content of chymotrypsin mRNA and, to a lesser extent, in that of amylase mRNA, suggesting that the increase in the mRNA levels most likely resulted from transcriptional control of the corresponding genes and/or changes in mRNA stability. We were also able to show that feeding a protein-free diet for a 13-day period actually resulted in a significant enhancement of the relative levels of the anionic isoenzymic forms of serine proteinases, whereas those of the cationic forms were decreased. A similar effect had been reported as early as 1968 by Vandermeers *et al.* [20] and, more recently, by Schick *et al.* [1], though under significantly different conditions in both cases.

In the present study, specific oligonucleotide probes were used to investigate the effect of a high-carbohydrate protein-free diet on the levels of specific mRNAs coding for the cationic form and both anionic forms of trypsin, as well as for anionic and cationic elastases.

MATERIALS AND METHODS

Materials

[α-^{32}P]dCTP and [α-^{32}P]dATP (111 TBq/mmol) were purchased from Amersham Corp. (Les Ulis, France). Formamide (spectrograde) was from Eastman Kodak Co. (Rochester, NY, U.S.A.) and formaldehyde from Fluka AG (Basel, Switzerland). Nitrocellulose sheets were from Schleicher und Schüll (Dassel, Germany). The nick-translation kit and RNA ladder (0.16–1.77 kb) were from BRL (Cergy-Pontoise, France). All the other reagents were as indicated in [19].

Animals and diets

The detailed compositions of the equal-energy ('isocaloric') standard diet (23% casein/58% carbohydrate) and protein-free diet (0% casein/81% carbohydrate) have been reported previously [19]. Control and experimental diets were administered to groups of five male Wistar rats (250–300 g) for various periods of time (0–5 days). Rats were then killed by cervical dislocation and the pancreatic glands were rapidly removed by surgical excision.

Dot-blot and Northern-blot analyses of RNA

Preparation of total RNA and subsequent denaturation in water/formamide (1:1, v/v) was carried out as indicated in [19]. In some experiments, however, RNA was denatured in a 20% (v/v) formaldehyde solution containing 1.5 M-NaCl, 0.1 M-NaH_2PO_4, 0.01 M-EDTA, pH 7.4, for 15 min at 60 °C, and finally dotted on nitrocellulose with a manifold (Schleicher und Schüll). In other experiments, after solubilization of RNA samples in formaldehyde and heat denaturation for 5 min at 95 °C, RNA was separated by electrophoresis (80 V, 4 h) on horizontal 1.2%-agarose gel plates in a 40 mM-Mops buffer, pH 7.0 [21]. The running gel was then rinsed and blotted on to a nitrocellulose

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filter [22]. All filters were baked at 80 °C in a vacuum oven for 90 min [23].

Specific pancreatic cDNA clones

The cDNA encoding anionic trypsin was obtained from MacDonald *et al.* [14].

Synthesis and purification of oligonucleotides

The following 31-mer oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method with an automatic DNA synthesizer (Applied Biosystems 381 A):

A_I 5′ > GACACACTGATTGAAGAGAGACTGAAGATAT < 3′
A_{II} 5′ > GACATAATGACTGTAGAGGGATTGGAGAGCT < 3′
Cat 5′ > TGACTACAAAGTATAAAGACATAAAGGCGTT < 3′
E_I 5′ > CTTGGGGAGGCCACTGGACTCAGGAAGACGT < 3′
E_{II} 5′ > CCCAAGGTGACAGTCACAGAGATGCCTTTGC < 3′
cA_I 5′ > ATATCTTCAGTCTCTCTTCAATCAGTGTGTC < 3′
cA_{II} 5′ > AGCTCTCCAATCCCTCTACAGTCATTATGTC < 3′
cCat 5′ > AACGCCTTTATGTCATTATACTTTGTAGTCA < 3′

Oligonucleotidic sequences A_I and A_{II} were complementary to the 3′-non-coding region of the mRNAs specific for anionic trypsinogens I and II [14] respectively, whereas sequence Cat is complementary to that of cationic trypsinogen [13]. Oligonucleotides E_I and E_{II} correspond to the 3′ part of the mRNAs coding for anionic elastase I and cationic elastase II respectively [2]. Oligonucleotide sequences cA_I, cA_{II} and cCAT correspond to the 3′ non-coding region of the mRNAs coding for anionic trypsins I and II, and cationic trypsin, respectively, i.e. are complementary to A_I, A_{II} and Cat oligonucleotides. All the synthetic nucleotide sequences were analysed by using the algorithm program [23a] in the GenBank (Release 54; December 1987), and their respective specificity was shown to be exclusively restricted to the corresponding region of the selected mRNAs. Synthetic oligonucleotides were separated from reaction by-products by electrophoresis on a 20%-(w/v)-polyacrylamide gel. The bands corresponding to the 31-mer oligonucleotides were excised and further purified on a C_{18} Sep-Pak (desalting) column as indicated in [24].

Hybridization experiments with labelled trypsin cDNA probe [specific radioactivity $(0.2–1.0) \times 10^9$ c.p.m./μg] were carried out under previously described experimental conditions [19], the probe being added to the hybridization buffer at $(0.5–1.0) \times 10^7$ c.p.m./ml. When oligonucleotides were used, they were 5′-end-labelled with T_4-phage polynucleotide kinase and [γ-^{32}P]dATP [25] and used for hybridization at $(2.0–2.5) \times 10^7$ c.p.m./ml of buffer. Prehybridization, hybridization and washing conditions were those of Mueckler *et al.* [26]. Radioactive filters were then exposed to X-ray films (RX from Fuji) and analysed by autoradiography. The radioactivity contained in individual RNA dots was counted in a Beckman LS 3800 liquid-scintillation spectrometer.

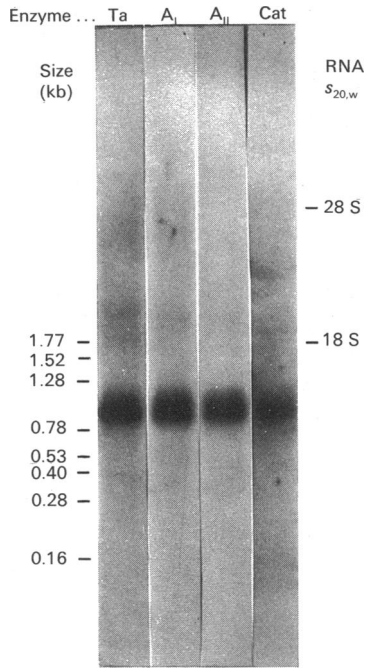

Fig. 1. Northern-blot hybridization experiments with radiolabelled synthetic oligonucleotides

After denaturation of total pancreatic RNA from an adult rat and fractionation on a 1.2%-agarose gel in 2.2 M-formaldehyde, RNA samples were transferred to nitrocellulose paper. Filters were then hybridized with the nick-translated cDNA clone coding for anionic trypsin (Ta) or the 5′-labelled synthetic oligonucleotides corresponding to anionic trypsins I (A_I) and II (A_{II}) and to cationic trypsin (Cat). RNA sizes in kb are indicated to the left, and the migration of 18 S and 28 S RNA is shown to the right.

RESULTS

Quantification of the respective levels of mRNAs coding for anionic trypsins I and II, and cationic trypsin, on the one hand, and for anionic and cationic elastases on the other hand, was carried out by hybridization with the radiolabelled specific 31-mer oligonucleotides. However, in order to assess the specificity of the synthetic oligonucleotides, they were first hybridized to Northern blots of total pancreatic RNA. Fig. 1 shows that a single hybridization band, the migration rate of which corresponded to a 0.85 kb mRNA, was detected whatever the synthetic oligonucleotide used. Interestingly, the migration rate of the mRNAs specific for both anionic trypsins and the cationic trypsin was identical with that of the anionic trypsin mRNA as detected with the corresponding specific cDNA probe [14].

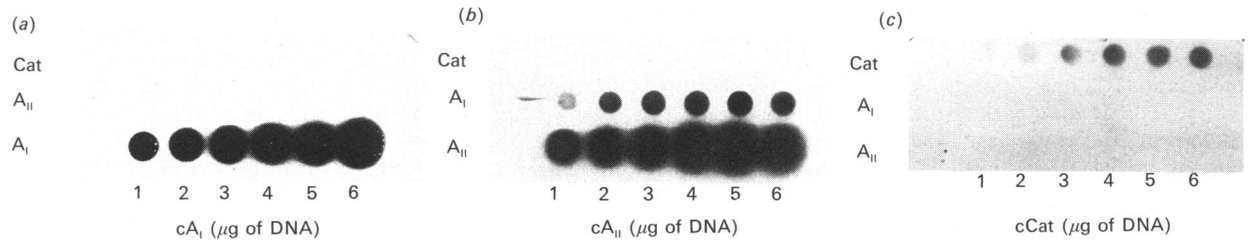

Fig. 2. Cross-hybridization of the 3′-regions of trypsin mRNA with radiolabelled complementary oligonucleotides

31-mer oligonucleotides complementary to probes A_I, A_{II} and Cat [cA_I (*a*), cA_{II} (*b*) and cCat (*c*)] corresponding to the coding region of trypsinogen mRNAs were synthesized, denatured and spotted (1–6 μg) on to the nitrocellulose paper. The filters were hybridized with 5′-labelled oligonucleotides A_I, A_{II} and Cat. The filters were exposed to X-ray film for 20 h at −70 °C.

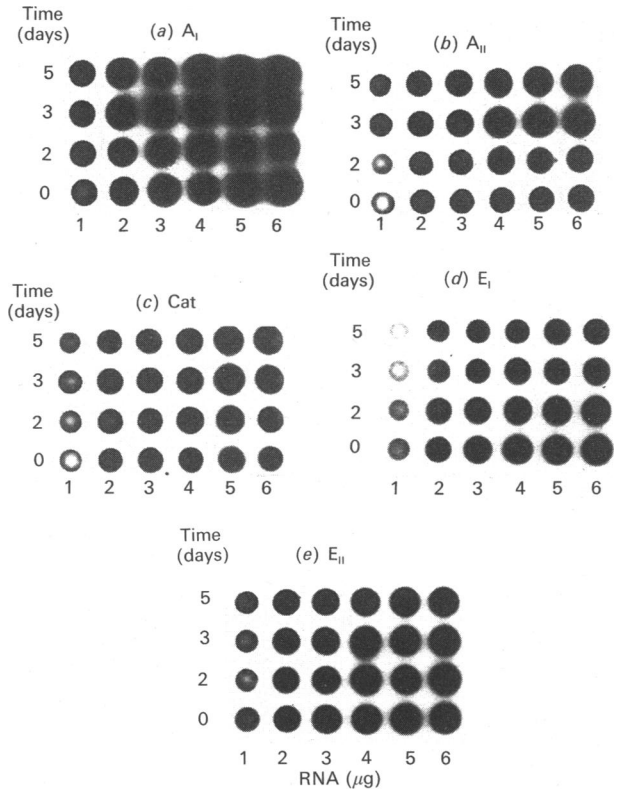

Fig. 3. Quantification of mRNA by dot-blot hybridization

Total pancreatic RNA isolated from rats fed the protein-free diet for 0, 2, 3 and 5 days was denatured, and the resulting samples (1–6 μg) were spotted on to a nitrocellulose filter, baked, and hybridized with the 5′-labelled oligonucleotides as indicated: (*a*) A_I, anionic trypsin I; (*b*) A_{II}, anionic trypsin II; (*c*) Cat, cationic trypsin; (*d*) E_I, anionic elastase; and (*e*) E_{II}, cationic elastase. Each spot was cut out and its radioactivity counted to estimate the level of each mRNA.

In another set of experiments, oligonucleotide sequences complementary to those of probes A_I, A_{II} and Cat (sequences cA_I, cA_{II} and cCat) were fixed on to nitrocellulose and hybridized with each of the three radiolabelled probes A_I, A_{II} and Cat. Fig. 2 shows that cross-hybridization was detectable only between sequences A_I and cA_{II}. However, it was only 3.7% of that observed with sequence A_{II}. It was important to check the specificity of trypsin probes, since anionic-trypsin-I and -II oligonucleotides (sequences A_I and A_{II}) are 70% identical. As far as elastase oligonucleotides are concerned, the identity does not exceed 29%.

Once the specificity of each probe was established, they were used in dot-blot hybridization experiments in order to determine the levels of anionic- or cationic-enzyme mRNAs specific for trypsin and elastase. The representative autoradiograms are shown in Fig. 3, and the corresponding quantitative results are given in Table 1. It should first be emphasized that trypsin I mRNA has been reported to be a major pancreatic mRNA in the adult rat, accounting for 2–5% of the total messenger pool, whereas trypsinogen II mRNA is present in at least 20-fold lower amounts [14]. In our experiments we found that the amount of mRNA hybridized by the trypsinogen II oligonucleotide probe (A_{II}) represented as much as 25% of that hybridized by the trypsinogen I oligonucleotide (A_I). The amount of mRNA hybridized by the cationic-trypsinogen oligonucleotide probe (Cat) represented less than 16% of that of anionic trypsinogen I mRNA, despite the fact that the level of cationic enzyme activity represented 50% of the anionic forms.

The effect of the protein-free carbohydrate-rich diet on trypsin mRNA levels is reported in Table 1, all the values being normalized to those found in control rats. As indicated, protein deprivation increased the concentration of the mRNAs coding for both anionic trypsins and for cationic trypsin. Here again, the maximal enhancement of mRNA levels was observed on the third day of dietary manipulation and persisted throughout the study. In contrast, no change in the concentrations of mRNAs specific for anionic and cationic elastases was observed during administration of the experimental diet. With respect to chymotrypsin mRNA, a pronounced increase in the corresponding messenger (about 4-fold) was previously measured [19] when animals were fed the protein-free diet for 3 days.

DISCUSSION

The present study describes the time-course response of mRNAs specific for a number of selected pancreatic enzymes during protein deprivation in the rat. It is therefore complementary to our previous report [19] on the accumulation of some specific proteins in pancreatic tissues under similar experimental conditions.

It has previously been reported [1,19] that feeding a protein-free diet for a 13-day period caused a marked decrease in the rate of biosynthesis of cationic serine proteinases and amylase, and an increase in that of anionic serine proteinases in the rat pancreas. However, the response of pancreatic enzymes to a protein-free carbohydrate-rich diet should be distinguished from the well-known nutritional adaptation, since the biosynthesis of amylase as well as that of anionic serine proteases, including trypsin, elastase and chymotrypsin was modified in an opposite way to their dietary substrates.

The physiological significance of increases in the biosynthesis of anionic serine proteinases is still not completely understood. A possible explanation would be that these enzymes are required to carry out digestion of endogenous proteins in the intestinal lumen. Whatever the reason may be, the main feature of this pancreatic regulation is the opposite response in the biosynthesis of anionic and cationic enzymes. It was therefore interesting to quantify the corresponding mRNAs. This was not possible with cDNA probes, on account of the high degree of identity existing between the nucleotide sequences of serine-proteinase isoenzymic forms. For example, it has recently been shown that the nucleotide sequence of rat cationic trypsinogen is 76 and 75% identical with those of rat anionic trypsinogens I and II respectively [13], and that the sequence identity between the two anionic zymogens is $\leqslant$ 88% [14]. The situation is probably even more complicated, owing to the existence of a large number of trypsin genes in the rat pancreas [12]. In addition, the two rat pancreatic elastases are known to display 58% amino acid sequence identity and to be coded for by two prominent mRNAs exhibiting 62% nucleotide sequence identity [27]. In contrast with the coding regions of pancreatic isoenzyme mRNAs, the 3′ non-coding regions have a lesser degree of sequence identity, which is, for example, 66% for anionic trypsins I and II [14]; 31-mer oligonucleotides complementary to the 3′ non-coding regions of trypsin mRNAs (a cationic and two anionic forms) and elastases (an anionic form and a cationic form) were therefore synthesized. The specificity of trypsin oligonucleotide was assessed by cross-hybridization with the complementary corresponding probes.

Protein deprivation was found to increase the levels of the mRNAs specific for anionic trypsins I and II and cationic trypsin by 2.3-, 2.8- and 2.3-fold respectively. The increase in anionic-trypsin mRNAs correlated well with the 1.86–2.81-fold enhancement of the synthesis of both anionic trypsin forms [1], suggesting that protein deprivation affects the synthesis and/or

Table 1. Relative levels of the specific mRNAs coding for trypsins and elastases in response to protein deprivation

mRNA levels were calculated from dot-blot experiments on total pancreatic RNA with the corresponding oligonucleotide probes: A_I, A_{II}, Cat, E_I and E_{II} (see the Materials and methods section). Each value is the mean ± S.D. for five rats normalized to that of control rats on day 0.

	Trypsin			Elastase	
	Anionic		Cationic	Anionic	Cationic
Consumption period (days)	A_I	A_{II}	Cat	E_I	E_{II}
0	1.00	1.00	1.00	1.00	1.00
2	1.23±0.20	1.32±0.19	1.35±0.18	1.09±0.37	1.03±0.21
3	2.29±0.21	2.81±0.43	2.21±0.11	0.93±0.46	1.27±0.17
5	1.70±0.36	2.24±0.80	2.34±0.23	0.89±0.53	1.12±0.34

stability of the corresponding messengers. In contrast, a 3-fold decrease in the synthesis of trypsinogen 3 (cationic) in the pancreas of rats fed a protein-free diet has been reported, whereas we observe a 2.3-fold increase in the level of the cationic-enzyme mRNA under similar conditions. Although the existence of a mechanism regulating the translation of the cationic enzyme mRNA could explain these results, the fact that our synthetic probe corresponds to a single form of cationic trypsin may account for this discrepancy. The cationic trypsinogen corresponding to our synthetic oligonucleotide probe actually represented less than 16% of the amount of anionic trypsinogen I mRNA, whereas the level of the major cationic enzyme, namely trypsinogen 3, was shown to be about one-half that of the major anionic trypsinogen 1 isoenzyme in the tissue of control rats. Thus the cationic-trypsin mRNA that was investigated throughout this study is not that corresponding to the major cationic-trypsin form and could be affected differently from the major cationic trypsin mRNA by protein deprivation.

On the other hand, it is worth stressing that no change in the content of mRNAs specific for elastase I and II was observed in pancreatic tissue in response to the protein-free diet. Since under comparable protein-deprivation conditions, Schick *et al.* [1] have reported a 2.2-fold increase in biosynthesis of anionic elastase I and a 3-fold decrease in that of cationic elastase, the regulation of gene expression in this case could be due to translation control of the specific mRNAs.

Taken together, our results indicate that the levels of different serine-proteinase mRNAs are modulated in different ways by diet. Although there was no significant effect of the protein-free diet on the level of mRNAs coding for both elastase forms, an important increase in that corresponding to the three trypsin forms was observed. Trypsin mRNA accumulation probably results from transcriptional control and/or change in mRNA stability. In contrast, elastase expression is translationally regulated. Although this study indicates the existence of a variety of control sites in pancreatic enzyme production, the biochemical mechanisms by which the regulation of expression of the corresponding genes occurs are still far from being well understood.

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REFERENCES

1. Schick, J., Verspohl, R., Kern, H. & Scheele, G. (1984) Am. J. Physiol. **247**, G611–G616
2. Swift, G. H., Dagorn, J. C., Ashley, P. L., Cummings, S. W. & MacDonald, R. J. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 7263–7267
3. Neurath, H., Walsh, K. A. & Winter, W. P. (1967) Science **158**, 1638–1644
4. De Haen, C., Neurath, H. & Teller, D. C. (1975) J. Mol. Biol. **92**, 225–259
5. Tani, T., Ohsumi, J., Mita, K. & Takiguchi, Y. (1988) J. Biol. Chem. **263**, 1231–1239
6. Shirasu, Y., Takemura, K., Yoshida, Y., Sato, Y., Iijima, H., Shimada, Y., Mikayama, T., Ozawa, T., Ikeda, N., Ishida, A., Tamai, Y., Matsuki, S., Tanaka, J., Ikenaga, H. & Ogawa, M. (1988) J. Biochem. (Tokyo) **104**, 259–264
7. Swift, G. H., Craik, G. S., Stary, S. J., Quinto, C., Lahaie, R. G., Rutter, W. J. & MacDonald, R. J. (1984) J. Biol. Chem. **259**, 14271–14278
8. Guy, O., Lombardo, D., Nartlett, D. C., Amic, J. & Figarella, C. (1978) Biochemistry **17**, 1669–1675
9. Louvard, M. N. & Puigserver, A. (1974) Biochim. Biophys. Acta **371**, 177–185
10. Ohlsson, K. & Tegner, H. (1973) Biochim. Biophys. Acta **317**, 328–337
11. Brodrick, J. W., Largman, C., Geokas, M. C., O'Rourke, M. & Ray, S. B. (1980) Am. J. Physiol. **289**, G511–G515
12. Craik, C. S., Choo, Q. L., Swift, G. H., Quinto, C., MacDonald, R. J. & Rutter, W. J. (1984) J. Biol. Chem. **259**, 14255–14264
13. Fletcher, T. S., Alhadeff, M., Craik, C. S. & Largman, C. (1987) Biochemistry **26**, 3081–3086
14. MacDonald, R. J., Stary, S. J. & Swift, G. H. (1982) J. Biol. Chem. **257**, 9724–9732
15. Ben Abdeljlil, A. & Desnuelle, P. (1964) Biochim. Biophys. Acta **81**, 136–149
16. Reboud, J. P., Marchis-Mouren, G., Pasero, L., Cozzone, A. & Desnuelle, P. (1966) Biochim. Biophys. Acta **17**, 351–367
17. Corring, T. (1977) World Rev. Nutr. Diet. **27**, 132–144
18. Poort, S. R. & Poort, C. (1980) Biochim. Biophys. Acta **81**, 136–149
19. Dakka, N., Wicker, C. & Puigserver, A. (1988) Eur. J. Biochem. **176**, 231–236
20. Vandermeers, A., Khayert, M. H., Rathé, J. & Christophe, J. (1968) Biochim. Biophys. Acta **158**, 448–455
21. Lehrach, H., Diamond, D., Wozney, J. M. & Boedtker, H. (1977) Biochemistry **16**, 4743–4751
22. Southern, E. M. (1975) J. Mol. Biol. **98**, 503–517
23. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 5201–5205
23a. Dumas, J. P. & Ninio, J. (1982) Nucleic Acids Res. **10**, 197–206
24. Atkinson, T. & Smith, M. (1984) in Oligonucleotide Synthesis: A Practical Approach (Gait, M. J., ed.), pp. 35–81, IRL Press, Oxford
25. Richardson, C. C. (1971) in Procedures in Nucleic Acid Research (Cantoni, G. L. & Davies, D. R., eds.), vol. 2, pp. 815–818, Harper and Row, New York
26. Mueckler, M. H., Merrill, M. J. & Pitot, H. C. (1983) J. Biol. Chem. **258**, 6109–6114
27. MacDonald, R. J., Swift, G. H., Quinto, C., Swain, W., Pictet, R. L., Nikovits, W. & Rutter, W. J. (1982) Biochemistry **21**, 1453–1463

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