Synthesis and maturation of recombinant human tumor necrosis factor in eukaryotic systems

Rita Müller*, Anne Marmenout* and Walter Fiers+

Biogent, Jozef Plateaustraat 22 and *+Laboratory of Molecular Biology, State University of Ghent, K.L. Ledeganckstraat 35, Ghent, Belgium*

Received 24 December 1985

The biosynthesis of human tumor necrosis factor (hTNF) was studied. The amino-terminal extension of the hTNF precursor (26 kDa polypeptide) was not cleaved off in a cell-free system supplemented with dog pancreas microsomes. Correct maturation of pre-hTNF was nevertheless not restricted to the macrophage system: in the medium of a TNF-producing, transformed CHO cell line, a (weak) ~20 kDa, an ~18.5 kDa (doublet) and a 17 kDa TNF polyeptide, the latter corresponding to mature hTNF, were revealed by specific immunoprecipitation. Similar results were obtained with *Xenopus laevis* oocytes, injected with hTNF mRNA, except that the 20 kDa band was lacking. The results are discussed in relation to the secretion mechanism of hTNF.

Tumor necrosis factor *Protein synthesis* *Protein maturation* *(CHO cell,* Xenopus laevis*)* *Oocyte* *Reticulocyte lysate*

1. INTRODUCTION

Human tumor necrosis factor (hTNF), a cytokine produced by macrophages, has been cloned by several groups [1–4]. A comparison of its cDNA with the NH_2-terminal end of the mature protein secreted by induced U937 cells [1] or HL60 cells [2,4] indicated that it is synthesized as a precursor with an NH_2-terminal extension of 76 amino acids, while the mature product contains 157 amino acid residues [1,2,4]. Although atypically long, the presequence contains a central, highly hydrophobic region (position −46 to −21 [1,2]) and resembles in this respect to so-called signal peptide of many other secretory proteins [5]. Noteworthy however is the presence of Lys-Lys and Arg-Arg sequences among the first 30 amino acid residues of the presequence [1–4] which suggests that the NH_2-terminal extension might perhaps be cleaved to peptides with special biological functions, since pairs of basic amino acids are often targets for the release of such peptides [6–8]. Here we report that the 'signal' peptide of hTNF is not removed when its messenger RNA is translated in a cell-free system supplemented with dog pancreas microsomes, a phenomenon which sets it apart from many other secretory proteins [5]. We also show that the hTNF precursor can be properly matured in CHO cells and *Xenopus laevis* oocytes and that, consequently, its special maturation is not restricted to the macrophage system.

* Present address: Innogenetics N.V., Industriepark Zwijnaarde 7, Bus 4, 9710 Zwijnaarde, Belgium

2. MATERIALS AND METHODS

2.1. *Construction of plasmids*

The information for almost the entire hTNF messenger RNA (from the *Fok*I site at position 59 to the *Dra*I site at position 1511) was inserted into plasmid pSP65 (Promega-Biotech) in two steps.

Plasmid pSP65 contains a promoter of *Salmonella* phage SP6 and allows one, by means of SP6-RNA polymerase (Promega-Biotech), to transcribe selectively any gene of interest in vitro [9]. The

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

cDNA clone phTNF1 [1] was first digested with *Fok*I, and blunted by means of Klenow enzyme (Boehringer). The *Fok*I fragment (blunted) from position 59 to 856 of the cDNA was purified and inserted into the *Sma*I site of pSP65. The orientation of the insert was verified by restriction enzyme analysis. The resulting plasmid was consequently digested with *Apa*I (position 228 of the hTNF cDNA) and *Hin*cII and ligated with the *Apa*I-*Dra*I fragment of phTNF1 (position 228–1511 of the cDNA). The construction containing the cDNA in the correct orientation was designated pSP65-hTNF1 (fig.1).

Plasmid pSVd2-hTNF (*Pst*I)-1 (fig.1) contains the entire coding region of the hTNF gene as a *Pst*I fragment originating from the cDNA clone phTNF1 [1]. Insertion was carried out by means of *Sal*I linkers into the unique *Sal*I site of pSVd2-3tss$^+$, a modified pSVd2 vector [10]. The expression unit of this vector consists of the SV40 early promoter, the *Sal*I site, the SV40 small t-antigen splice donor and acceptor sites and the SV40 polyadenylation site.

2.2. *In vitro synthesis of capped transcripts*

Capped mRNA was synthesized in vitro as described by Contreras et al. [11]. Plasmid pSP65-hTNF1 linearized at the unique *Nco*I site was used as a template for run-off transcription. In vitro transcription was carried out as recommended by the supplier (Promega-Biotech) except that the reaction mixture contained 0.5 A_{260} units of $m^7(G^5)ppp(5')G$ cap structure (Pharmacia PL-Biochemicals), 250 μM GTP and 500 μM of the 3 other NTPs (Boehringer). After 1 h at 40°C, the incubation mixture was phenol-chloroform extracted and the newly synthesized RNA precipitated with ethanol. No attempts were made to remove template DNA. The pellet was dissolved in H_2O to a final concentration of approx. 100 μg RNA/ml.

2.3. *Translation in cell-free system supplemented with dog pancreas microsomes*

Cell-free translation was carried out using a rabbit reticulocyte lysate (Amersham) and canine microsomal membranes (Amersham) essentially as recommended by the supplier. About 2–4 μl of in vitro synthesized capped transcript solution was used per 20 μl of lysate. A high specific activity [^{3}H]amino acid mixture (TRK 550 Amersham) was used for labeling. Prior to the addition of Laemmli sample buffer [12], lysates were incubated with 5 μl RNase A (200 μg/ml, Boehringer) for 15 min at 30°C.

2.4. *In vivo labeling of proteins*

CHO cells were cotransfected with pSVd2-hTNF1 (*Pst*)1 and pAdD26 SV(A)-3 according to the method of Scahill et al. [13]. TNF-producing colonies were subjected to methotrexate (10 mM) amplification [14] and assayed for TNF activity as described [15]. For labeling, subcultures of a high producer cell line (approx. 10000 units/ml) were grown to confluency in medium (Gibco no.

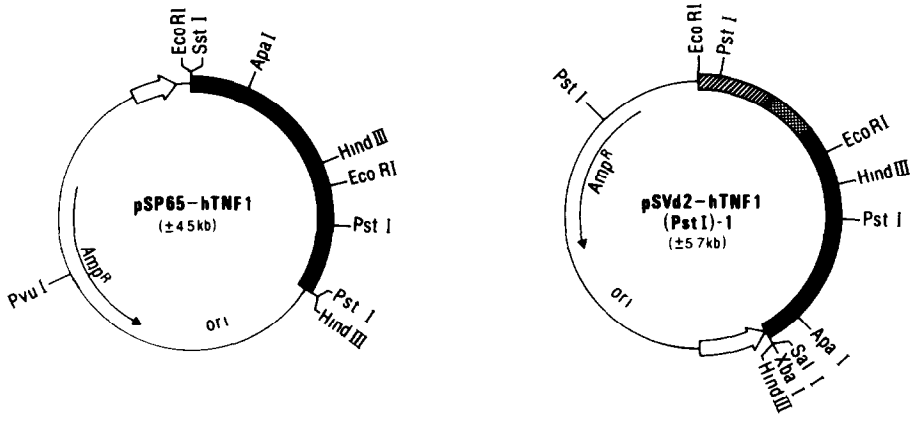

Fig.1. Plasmids pSP65-hTNF1 and pSVd2-hTNF (*Pst*I)-1. Only relevant restriction sites are shown. The black bar represents hTNF cDNA information. The SP6 promoter and the SV40 early promoter are indicated by an open, large arrow. The SV40 small t splice region and polyadenylation segment are represented by a stippled and a hatched box, respectively. Amp^R, ampicillin resistance.

0722000) supplemented with 10% fetal calf serum. Cells were split the following day. Labeling was done overnight in medium containing 10% dialysed fetal calf serum, 50 μCi/ml of a ^{14}C-labeled amino acid mixture (Amersham) and 20-fold reduced amounts of unlabeled amino acids.

X. laevis oocytes were injected with 50 nl of capped transcript solution (see above) per oocyte and incubated with 5 μl per oocyte of MBS (modified Barth solution) [16] supplemented with 100 μCi/ml of a ^{14}C-amino acid mixture (Amersham) for 24 h at 18°C.

2.5. *Immunoprecipitation*

Rabbit anti-hTNF serum (5 μl) and supernatant (0.5 ml) were mixed and incubated on ice for 2–3 h. For each μl of antiserum used, 10 μl of a 50% prewashed suspension of protein A-Sepharose (Pharmica) in phosphate-buffered saline (PBS) were added and the suspension was agitated gently at 4°C overnight. The beads were washed with several volumes of PBS containing 0.05% Tween 80 and the antigen-antibody complexes were eluted with either sample buffer or Tris-SDS buffer according to Schwyzer et al. [17].

2.6. *Gel electrophoresis and autoradiography*

Samples were electrophoresed on 15% SDS-polyacrylamide slab gels [12]. After staining (Serva blue R250) and destaining, gels were soaked in Amplify™ (Amersham) or Enlightning™ (New England Nuclear) for 30–60 min, washed twice with water and dried. Autoradiography was done at −70°C using Fuji X-ray film and intensifying screen.

3. RESULTS

3.1. *Translation in cell-free system supplemented with dog pancreas microsomes*

In vitro synthesized, capped transcripts of hTNF were translated in a rabbit reticulocyte lysate in the presence and absence of dog pancreas microsomes as described in section 2. The results, shown in fig.2, can be summarized as follows: in the absence of microsomes (lane 2) a 26 kDa polypeptide can be detected, a size which is in agreement with that expected for the precursor. The presence of a faint, faster migrating band might possibly be ascribed to a second start of translation initiation in vitro [18]:

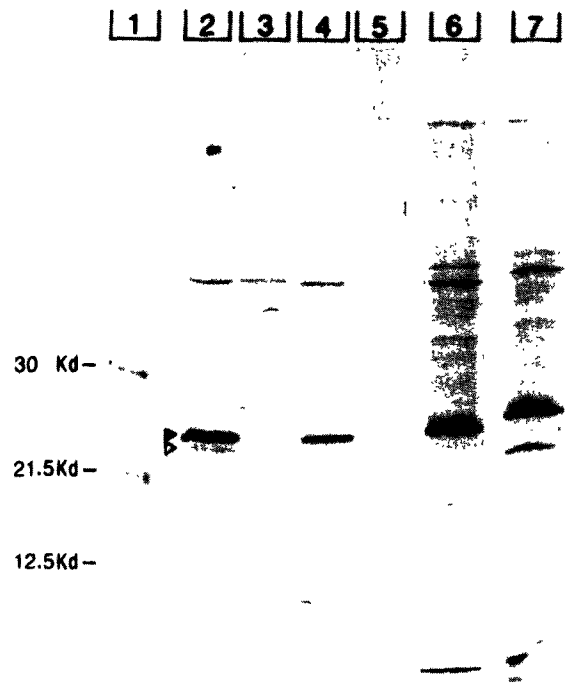

Fig.2. Translation products in a reticulocyte system in the presence and absence of dog pancreas microsomes. As described in section 2, in vitro synthesized, capped transcripts of the hTNF gene were translated in a rabbit reticulocyte lysate, all or not supplemented with dog pancreas microsomes. The ^{3}H-labeled translation products were electrophoresed on a 15% SDS-polyacrylamide gel which was further processed for autoradiography. Lanes: 1, [^{14}C]methylated protein mixture (Amersham) [carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), cytochrome *c* (12.5 kDa), aprotinin (6.5 kDa), insulin chain B (3.4 kDa) and chain A (2.35 kDa)]; 2, translation products with TNF transcript added but no microsomes; 3, control (no RNA, no microsomes added); 4, translation products obtained with TNF transcripts and microsomes added; 5, control (no RNA but microsomes present); 6, translation products in the presence of placental lactogen mRNA (Amersham) and no microsomes added; 7, as lane 6 but microsomes present. (►) Position of the 26 kDa hTNF precursor, (▷) a faster moving product, presumably resulting from internal initiation (cf. text).

indeed a second in-frame AUG is present only 5 codons following the first one [1–4]. In the presence of microsomes (lane 4) no faster moving bands can be detected although human placental lactogen (lanes 6 and 7) was properly processed in this system.

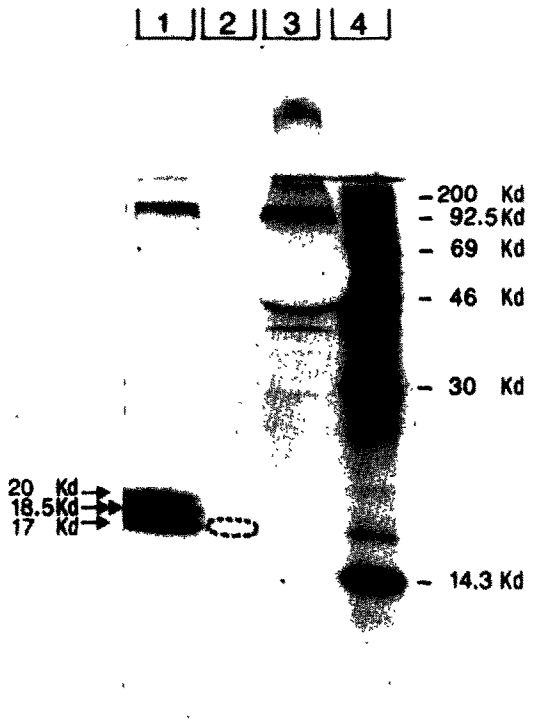

Fig.3. Analysis of TNF-specific products secreted by CHO cells. TNF-producing CHO cells were labeled with a ^{14}C-amino acid mixture for 19 h. The TNF related polypeptides were immunoprecipitated from the medium, analysed on a 15% SDS-polyacrylamide gel and the latter was processed for autoradiography as described in section 2. Lanes: 1, immunoprecipitate of the medium of ^{14}C-labeled TNF-producing CHO cells [cell line obtained by cotransfection with pSVd2-hTNF1 and pAdD26SV(A)-3]; 2, purified bacterially expressed mature hTNF Serva R250 stained (outlined in dashes); 3, control [immunoprecipitate from labeled CHO cells transfected with pSVd2 and pAdD26SV(A)-3]; 4, [^{14}C]-methylated protein mixture (Amersham) [myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa)].

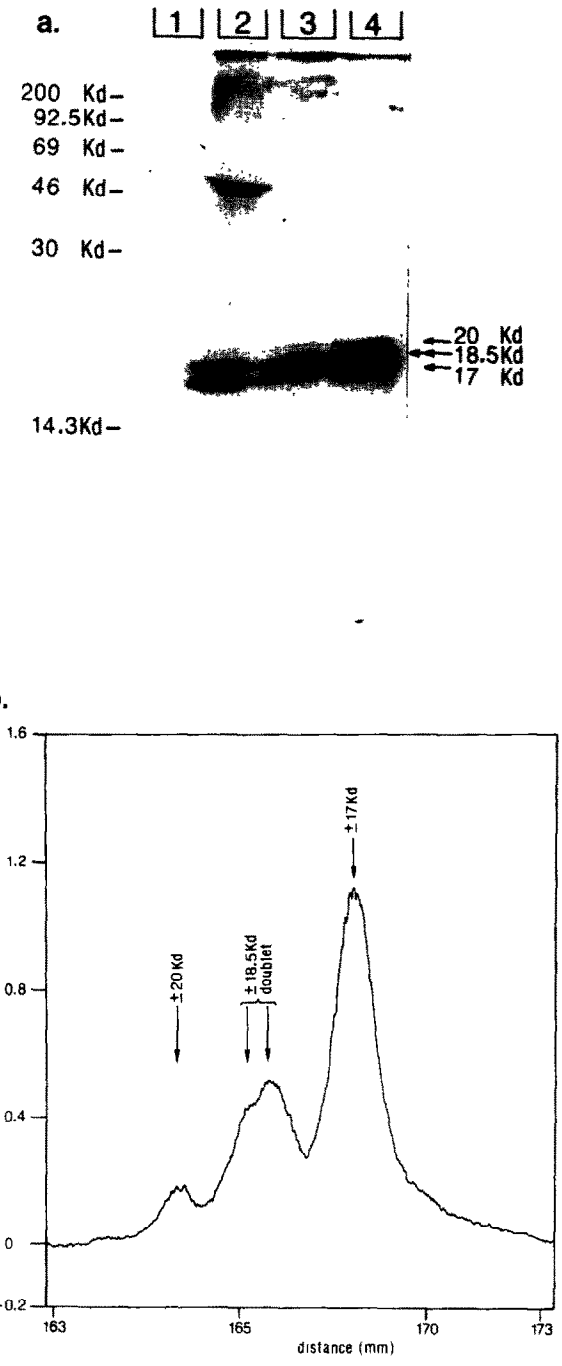

Fig.4. (a) Analysis of TNF-specific products secreted by *X. laevis* oocytes and by transfected CHO cells. *X. laevis* oocytes were injected, labeled and the bathing medium immunoprecipitated as described in section 2. The immunoprecipitate was loaded on a 15% SDS-polyacrylamide gel, which was further processed for autoradiography. Lanes: 1, immunoprecipitate of the bathing medium of labeled oocytes injected with H_2O; 2, as lane 1 but injected with in vitro synthesized capped transcripts of hTNF DNA; 3, immunoprecipitate of the medium of TNF-producing CHO cells (cf. fig.2) labeled with ^{14}C-amino acids for 7 h; 4, as lane 3 but labeled for 19 h. (b) Densitogram of the region of interest of lane 4, panel a. The doublet nature of the ~ 18.5 kDa band is clearly revealed.

3.2. *Translation in CHO cells*

CHO cells, transfected with pSVd2-hTNF (*Pst*I)-1, were labeled with a ^{14}C-amino acid mixture and TNF released into the medium was immunoprecipitated as described in section 2. Autoradiography of the SDS-polyacrylamide gel revealed the presence of 4 polypeptides: one of 20 kDa, a doublet at 18.5 kDa, and one of 17 kDa (fig.3, lane 1, see also fig.4b). The latter comigrates with purified bacterially expressed hTNF (lane 2), which in turn comigrates with natural TNF from U937 cells [1]. The doublet nature of the 18.5 kDa band is usually in evidence on the original autoradiograph (see also below).

3.3. *Translation in* X. laevis *oocytes*

X. laevis oocytes were injected with capped transcripts of hTNF and labeled in vivo with a ^{14}C-amino acid mixture as described in section 2. TNF in the bathing medium was immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (see also section 2).

As can be deduced from fig.4a, a pattern similar to that of the CHO cells was obtained except that the 20 kDa band was lacking (lane 2).

4. DISCUSSION

We have demonstrated that the signal peptide of hTNF is not cleaved in a cell-free translation system supplemented with dog pancreas microsomes, a finding which sets TNF apart from the secreted proteins studied so far. Proteins such as e.g. ovalbumin, rhodopsin, rat cytochrome P-450 and Sindbis envelope protein PE_2, which are also not cleaved in an in vitro translation system, are apparently secreted in toto [19–22].

The above finding, together with the presence of a hydrophobic region in the presequence which is long enough to span the membrane (i.e. 25 amino acid residues) suggests that TNF might be transported as an integral membrane protein, the mature protein being released subsequently at a late stage of secretion, e.g. in the secretory vesicles or even at the cell surface. In this respect, the finding of a membrane bound IL1 activity, in addition to the secreted one [23], might be relevant especially in view of the fact that IL1 is also a macrophage product. Further studies are needed to follow the biosynthetic pathway of mature TNF.

Our results however indicate that maturation of the TNF precursor is not restricted to the macrophage system. In the supernatant of CHO cells, transfected with pSVd2-hTNF (*Pst*I)-1, as well as in the supernatant of *X. laevis* oocytes injected with capped TNF transcripts a polypeptide can be detected which comigrates with bacterially expressed mature hTNF (fig.4a, lanes 2 and 3), which in turn comigrates with natural TNF from U937 cells [1]. Furthermore, the pSVd2-hTNF (*Pst*I)-1 transfected CHO cells secrete a TNF biological activity of approx. 10 000 units/ml, an activity which exceeds the one obtained with U937 cells after optimum stimulation [24]. The biological activity obtained in the supernatant of *X. laevis* oocytes injected with in vitro synthesized capped transcripts is also relatively high (in the range of 500 units/ml). Although precise quantitation is difficult, it seems that the specific activity of the TNF produced by these non-macrophage systems is about the same as the one of natural TNF obtained from an induced macrophage cell line. So far, we have no precise knowledge about the nature of the slower migrating polypeptides which are also present in the supernatants (~20 kDa and ~18.5 kDa doublet for CHO cells, ~18.5 kDa doublet only for *X. laevis* oocytes). Several possibilities can be envisaged: either these polypeptides are the result of secondary modification of the mature polypeptide or they represent intermediate precursor forms. Even a combination of both events cannot be excluded. The slower moving polypeptides are clearly not due to *N*-glycosylation since they are resistant to concentrations of endoglycosidase F which are sufficient to deglycosylate interferon (not shown). This result is in agreement with the predicted amino acid sequence for TNF [1] containing no potential *N*-glycosylation sites. Although not tested, we consider it very unlikely that they result from e.g. phosphorylation or acetylation since these modifications, unless very extensive, usually do not cause drastic changes in mobility. Nevertheless, it is not excluded that at least one of the polypeptides of the ~18.5 kDa doublet might be due to some form of modification, for example *O*-glycosylation, since in the supernatant of induced U937 cells a faint 19 kDa polypeptide can be detected which has the same N-terminal amino acid sequence as the predominant 17 kDa TNF

polypeptide (Smart, J. and Mattaliano, R. unpublished). Only a thorough characterization of the slower moving bands will definitely establish whether *O*-glycosylation has occurred and this might possibly shed more light on the mechanism of secretion also. A likely hypothesis is that at least one of the polypeptides of the 18.5 kDa doublet, and perhaps also the 20 kDa band, represent an intermediate precursor form. The presence of 21 amino acids between the end of the hydrophobic, presumably membrane-spanning, region in the presequence and the N-terminal end of the mature product supports this hypothesis of one or two intermediate precursor forms being about 1.5 and/or 3 kDa extended at the N-terminus.

Some additional evidence for this possibility is provided by lymphotoxin of which two forms are identified: a so-called mature form and one having 23 additional amino acids at the N-terminus [2]. If similar results could be confirmed for TNF, the model of hTNF being transported as an integral membrane protein would definitely be strengthened since the presence of immature forms in the supernatant could readily be explained by maturation at a late stage in the process.

ACKNOWLEDGEMENTS

We thank Dr Jan Tavernier for the construction of pSVd2-hTNF1 and José Van der Heyden for the transfection and maintenance of CHO cells. We are also grateful to Christine Vermeire and Wim Drijvers for their editorial and artistic contribution, respectively. Biogent is a subsidiary of Biogen S.A., Geneva.

REFERENCES

[1] Marmenout, A., Fransen, L., Tavernier, J., Van der Heyden, J., Tizard, R., Kawashima, E., Shaw, A., Johnson, M.-J., Semon, P., Müller, R., Ruysschaert, M.-R., Van Vliet, A. and Fiers, W. (1985) Eur. J. Biochem. 152, 515–522.

[2] Pennica, D., Nedwin, G.E., Hayflick, J.S., Seeburg, P.H., Derynck, R., Palladino, M.A., Kohr, W.J., Aggarwal, B.B. and Goeddel, D.V. (1984) Nature 312, 724–729.

[3] Shirai, T., Yamaguchi, H., Ito, H., Todd, C.W. and Wallace, R.B. (1985) Nature 313, 803–806.

[4] Wang, A.M., Creasy, A.A., Ladner, M.B., Lin, L.S., Strickler, J., Van Arsdell, J.N., Yamamoto, R. and Mark, D.F. (1985) Science 228, 149–154.

[5] Blobel, G., Walter, P., Chang, C.N., Goldman, B.M., Erickson, A.H. and Lingappa, V.R. (1979) in: Secretory Mechanisms (Hopkins, C.R. and Duncan, J.C. eds) pp. 9–36, Cambridge University Press, Cambridge.

[6] Noda, H., Furutami, Y., Takahashi, H., Toyasata, M., Hirose, T., Inayama, S., Nakanishi, S. and Numa, S. (1982) Nature 295, 202–206.

[7] Gubler, U., Seeburg, P., Hoffman, B.J., Gage, L.P. and Udenfriend, S. (1982) Nature 295, 206–208.

[8] Amara, S.G., Jonas, V., Rosenfeld, M.G., Oug, E.S. and Evans, R.M. (1982) Nature 298, 240–244.

[9] Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. 12, 7035–7056.

[10] Mulligan, R. and Berg, P. (1980) Science 209, 1422–1427.

[11] Contreras, R., Cheroutre, H., Degrave, W. and Fiers, W. (1982) Nucleic Acids Res. 10, 6353–6361.

[12] Laemmli, U.K. (1970) Nature 227, 680–685.

[13] Scahill, S.J., Devos, R., Van der Heyden, J. and Fiers, W. (1983) Proc. Natl. Acad. Sci. USA 80, 4654–4658.

[14] Kaufman, R.J. and Sharp, P.A. (1982) J. Mol. Biol. 159, 601–621.

[15] Ruff, M.R. and Gifford, R.E. (1981) in: Lymphokines (Pick, E. ed.) vol. 2, pp. 235–275, Academic Press, NY.

[16] Colman, A. (1985) in: Transcription and Translation, a Practical Approach (Hames, B.D. and Higgins, S.J. eds) pp. 49–69, IRL Press, Washington DC.

[17] Schwyzer, M., Weil, R., Frank, G. and Zuber, H. (1980) J. Biol. Chem. 255, 5627–5634.

[18] Hunt, T. (1985) Nature 316, 580–581.

[19] Palmiter, R.D., Gagnon, J. and Walsh, K.A. (1978) Proc. Natl. Acad. Sci. USA 75, 94–98.

[20] Schechter, I., Bernstein, Y., Zemell, R., Ziv, E., Kantor, F. and Papermaster, D. (1979) Proc. Natl. Acad. Sci. USA 76, 2654–2658.

[21] Bar-Nun, S., Kreibich, G., Adesnik, M., Alterman, L., Negishi, M. and Sabatini, D. (1980) Proc. Natl. Acad. Sci. USA 77, 965–969.

[22] Bonatti, S. and Blobel, G. (1979) J. Biol. Chem. 254, 12261–12264.

[23] Kurt-Jones, E.A., Beller, D.I., Mizel, S.B. and Unanue, E.R. (1985) Proc. Natl. Acad. Sci. USA 82, 1204–1208.

[24] Tavernier, J., Fransen, L., Marmenout, A., Van der Heyden, J., Müller, R., Ruysschaert, M.-R., Van Vliet, A., Bauden, R. and Fiers, W. (1986) in: Lymphokines (Pick, E. ed.) Academic Press, NY, in press.