Volume 245, number 1,2, 17–20 FEB 06845 March 1989

Prothymosin α is a nuclear protein

Julian D. Watts, Peter D. Cary and Colyn Crane-Robinson

Biophysics Laboratories, Portsmouth Polytechnic, White Swan Road, Portsmouth PO1 2DT, England

Received 22 December 1988

The cellular location of the so-called 'thymic hormone' prothymosin α has been studied by microinjection into the cytoplasm of *Xenopus* oocytes, followed by separate monitoring of nuclear and cytoplasmic concentrations. It is shown that prothymosin α migrates to the nucleus at a rate comparable to that of histone H1. Prothymosin α cannot therefore be a hormone in the usual sense of the word.

Prothymosin α; Thymosin; Nuclear targeting

1. INTRODUCTION

A peptide of 28 residues, named thymosin α_1, originally isolated from calf thymus [1] has been reported to have immuno-restorative properties and to offer protection against opportunistic infections [2]. It is frequently referred to as a 'thymic hormone' since significant levels have been reported in serum [3]. For a review see Low and Goldstein [4]. More careful extraction of calf thymus leads to the isolation of a longer peptide of 110 amino acids, prothymosin α, but little thymosin α_1 [5]. Prothymosin α includes thymosin α_1 as its 28 N-terminal amino acids. Since prothymosin α is reported to protect mice against opportunistic infection at much lower doses than thymosin α_1 [6], the latter is probably a proteolytic product of prothymosin α, derived largely from the extraction procedures used. The amino acid sequences of the rat, human and bovine prothymosin α proteins have been determined [7–9]. They are all very closely related and contain ~50% acidic residues, of which 24 are in a central section, broken only by interspersed glycine residues. In bovine prothymosin α there are only 7 large hydrophobic and no aromatic residues, suggesting that prothymosin α is not a folding protein.

A hormonal function for prothymosin α has been thrown into doubt, firstly by the demonstration, using cDNA cloning, that there is no signal peptide in the primary translation product [10,11], and also from the observation of a broad tissue distribution for the protein [12]. An intracellular function for prothymosin α, related to cell growth, is suggested by the observation that its mRNA level rises sharply in stimulated lymphocyte cultures [10]. That this function might even be nuclear was proposed on the basis of a putative nuclear localisation sequence near the C-terminus of prothymosin α [13].

In the experiments reported here, radioiodinated prothymosin α was used for cytoplasmic injection of *Xenopus laevis* oocytes to demonstrate that the protein indeed migrates to the nucleus.

Correspondence address: C. Crane-Robinson, Biophysics Laboratories, Portsmouth Polytechnic, White Swan Road, Portsmouth PO1 2DT, England

2. MATERIALS AND METHODS

Prothymosin α was purified from whole calf thymus cells by extraction with 5% perchloric acid followed by purification by ion-exchange chromatography on Sephadex CM-25, size fractionation on Sephadex G75 and finally by a further round of ion-exchange chromatography on Sephadex SPC-25 (Watts et al., in preparation).

Prothymosin α, histone H1 (a gift from Dr A. Thorne) and bovine serum albumin (BSA) (Sigma) were radio-iodinated as described [14]. Briefly, 150 μCi ^{125}I-labelled Bolton and Hunter

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50 © 1989 Federation of European Biochemical Societies

reagent (spec. act. 2000 Ci/mmol, Amersham) was dried under a stream of dry nitrogen and resuspended in 1 μl dimethylformamide (DMF). 1 nmol of protein (i.e. 12 μg prothymosin α, 20 μg histone H1 and 66 μg BSA) was dissolved in 19 μl of 100 mM sodium borate, pH 8.5, 50 mM NaCl and added to the DMF. Each sample was reacted on ice for 45 min, and the labelled protein separated from free radioactivity on a small column of Sephadex G25, previously flushed with 0.25% gelatin in running buffer (0.5% formic acid). Each sample was lyophilised, resuspended, counted and then diluted with unlabelled protein to give the required protein concentration and specific activity in 88 mM NaCl, 10 mM Tris, pH 7.5 (injection buffer).

A mature female *X. laevis* was killed and an ovary removed. Stage V–VI oocytes were separated from the membrane and maintained in modified Barth's saline at 18–19°C, as described [15]. Individual oocytes were injected into the vegetal hemisphere with 50 nl of the appropriate sample, 0.4 pmol/oocyte, and incubated in saline for varying lengths of time at 18°C. Ten oocytes were injected per time point. After fixing in 20% trichloroacetic acid (TCA) for 15 min at 18°C, the nuclei were removed using two pairs of watchmaker's forceps [16]. For the zero time point, oocytes were transferred to 20% TCA immediately after injection. Pooled nuclei and cytoplasms were counted using a Minaxi 5000 Auto-Gamma counter, resuspended in 50 μl of 10 M urea and insoluble material spun to the bottom of the tube. Aliquots were counted, diluted with 2 × SDS gel loading buffer, analysed by SDS gel electrophoresis [17], visualised with Coomassie and then dried down and subjected to autoradiography using pre-flashed film [18]. Densitometry of autoradiographs was performed on a Vitatron and peak areas obtained by weighing.

3. RESULTS

Xenopus oocytes were injected with iodinated prothymosin α, with histone H1 as a positive control [16,19] and with BSA as a non-migrating negative control. 10 oocytes were injected per protein for each time point, using 0.4 pmol of all 3 proteins per oocyte (5 ng in the case of prothymosin α). After incubation, oocytes were harvested by transfer to a 20% TCA solution. Following manual enucleation, pooled nuclei and cytoplasms were separately gamma counted and proteins were extracted by suspension in SDS gel loading buffer. Aliquots of the extracts were again gamma counted to correct for any differential protein extraction between nuclei and cytoplasms. Samples were loaded onto 15% polyacrylamide, 0.1% SDS gels, which were subsequently visualised by autoradiography against standard labelled prothymosin α.

One approach to analysis of the results is to follow the time course of radiolabel transfer to the nucleus by plotting the ratio of nuclear to cytoplasmic counts per oocyte. In order to reflect relative protein concentrations, the counts ratio was multiplied by 88/12, which represents the relative accessible volumes of cytoplasm to nucleus [16]. Fig.1 shows the results for an experiment that continued up to 48 h for prothymosin α. It appears that whereas BSA remains entirely in the cytoplasm, prothymosin α steadily accumulates in the nucleus. Despite some experimental variation, histone H1 is also seen to migrate to the nucleus.

A possible problem in following counts as a measure of migration is that any degradation of protein is not observed. Cytoplasmic degradation and loss of radiolabelled peptides from the oocytes would increase the apparent nuclear to cytoplasmic ratio. This does not appear to be a major problem since the prothymosin α counts per whole oocyte typically drop only by about 50% over 24 h. Nevertheless there is an advantage to monitoring intact protein on polyacrylamide gels. Fig.2 shows a 24 h time course of prothymosin α migration. Densitometry of the autoradiograph yields the relative amounts of nuclear and cytoplasmic protein, when corrected for the number of nuclei/

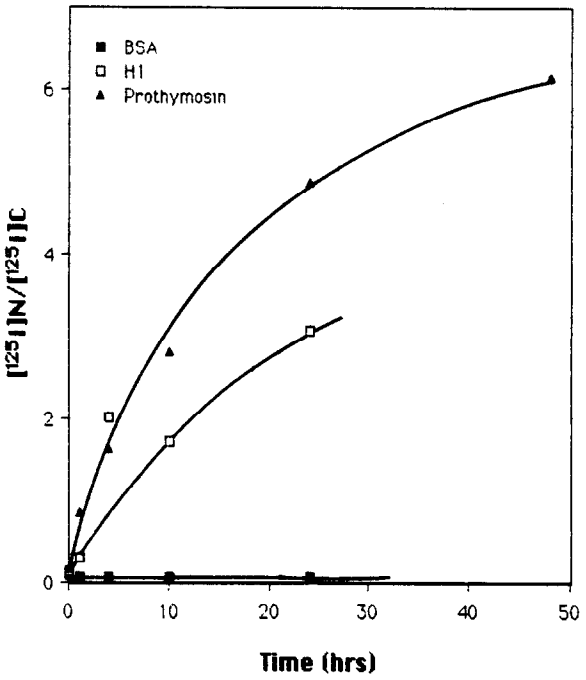


Fig.1. Time course of the relative nuclear to cytoplasmic concentration ratio of prothymosin α label. Histone H1 and BSA label are included for comparison.

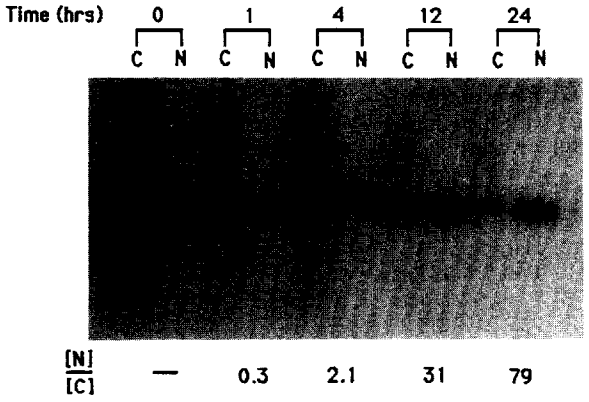


Fig.2. Autoradiograph of an SDS gel showing the time course of migration of prothymosin α from cytoplasm (C) to nucleus (N). The concentration ratio [N]/[C] was calculated from densitometry and taking into account the relative accessible volumes of cytoplasm and nucleus (88/12).

cytoplasms loaded per lane. The concentration ratio of intact prothymosin α was again obtained using the nuclear/cytoplasmic volume ratio of 12/88. The results are included in fig.2. Although the nuclear/cytoplasmic ratio for prothymosin α increases between 12 and 24 h, this is due to a decrease in the intensity of the cytoplasmic band rather than an increase in the nuclear band, which reaches a maximum by 12 h. There is therefore significant cytoplasmic degradation between 12 and 24 h and we conclude that migration is essentially complete within 12 h. Experiments continued up to 48 h show that the prothymosin α remains stable in the nucleus for this period (not shown).

An interesting feature of fig.2 concerns the radiolabelled minor components in the prothymosin α sample, which clearly react more readily with the Bolton and Hunter reagent than prothymosin α since they are not apparent in a Coomassie stained gel of the prothymosin α used for injection. Over the period in which the prothymosin α band appears in the nuclear lane, there is no sign of the impurity proteins. Even at 24 h they are still apparent in the cytoplasm. This indicates the specific nature of the prothymosin α migration.

4. DISCUSSION

Although a heterologous system is used here, injecting a bovine protein into *Xenopus* oocytes, the effectiveness of sequences in large T-antigen for stimulating migration in *Xenopus* oocytes has been demonstrated [20]. Bovine prothymosin α contains the sequence Asp-Thr-Lys-Lys-Gln-Lys-Thr-Asp, which includes a tetrapeptide similar to that in large T-antigen, Lys-Lys-Arg-Lys, defined as responsible for its nuclear migration [21]. Sequences similar or identical to the prothymosin α tetrapeptide are found in other nuclear proteins. For example, nucleolin contains the sequence Glu-Ala-Lys-Lys-Gln-Lys-Val-Glu which is identical to bovine prothymosin α within the tetrapeptide and likewise contains acidic residues located two amino acids away on either side. Although the present experiments do not show that this sequence is indeed that responsible for nuclear migration, they do lend support to the hypothesis of Gomez-Marquez and Segade [13] that this region of the protein can effect nuclear migration. Our result indicates that prothymosin α cannot be a hormone in the conventional sense of a secreted 'messenger' molecule.

A nuclear function for prothymosin α in transcription is suggested by its highly acidic nature and similarity to a number of other nuclear proteins. Proteins HMG14 and 17 both have acidic stretches, particularly towards their C-terminus, but this is not in the form of long contiguous stretches of acidic residues, as in prothymosin α. Nevertheless, HMG14 and 17 are of similar size to prothymosin α and are likewise non-folding proteins [22,23] and differ only in having a higher proportion of basic residues. Proteins HMG1 and 2, in contrast, each have a long contiguous acidic stretch at their C-terminus but differ from prothymosin α in having normally folded N-terminal domains [24,25]. HMG14 and 17 have been shown to be present on the nucleosomes of actively transcribed chromatin [26], whilst HMG1 and 2 are thought to bind to the linker DNA between nucleosomes [27] and may also serve to promote transcription [28,29]. The major nucleolar protein, nucleolin, also has several uninterrupted acidic stretches [30], the longest of which is 33 residues, not unlike the 30-residue long acidic tail of HMG1 [31]. Nucleolin has been postulated to play a role in both pre rRNA transcription and pre-ribosome assembly [32].

The activating domains of the yeast transcription factors GAL4 and GCN4 are also strongly

acidic, though they do not contain the contiguous sequences characteristic of HMG1/2 and prothymosin α [33,34]. These domains probably do not form defined tertiary structures.

An alternative functional role suggested by the highly acidic stretch in prothymosin α is one akin to that of *Xenopus* nucleoplasmin and nuclear protein N1 [35,36], i.e. as an assembly factor. Both of these *Xenopus* proteins include very acidic stretches but also contain folding regions, unlike prothymosin α.

Although nothing is known of the nuclear function of prothymosin α, a role in active chromosomal functions such as transcription or replication, is consistent with two observations: (i) the sharp rise in its mRNA on stimulation of quiescent cells, [10], and (ii) its virtual absence from non-dividing cells, e.g. brain, and its high level in tissues containing proliferative cells, such as thymus and spleen [12].

Acknowledgement: J.D.W. acknowledges the support of an SERC Postgraduate Studentship.

REFERENCES

[1] Goldstein, A.L., Low, T.L.K., McAdoo, M., McClure, J., Thurman, G.R., Rossio, J., Lai, C.-Y., Chang, D., Wang, S.-S., Harvey, C., Ramel, A.H. and Meienhofer, J. (1977) Proc. Natl. Acad. Sci. USA 74, 725–729.
[2] Caldarella, J., Goodall, G.J., Felix, A.M., Heimer, E.P. and Horecker, B.L. (1983) Proc. Natl. Acad. Sci. USA 80, 7424–7427.
[3] Weller, F.E., Mutchnik, M.G., Keren, D.F., Goldstein, A.L. and Naylor, P.H. (1985) J. Immunol. Methods 80, 45–53.
[4] Low, T.L.K. and Goldstein, A.L. (1986) Methods Enzymol. 116, 217–219 et seq.
[5] Haritos, A.A., Goodall, G.J. and Horecker, B.L. (1984) Proc. Natl. Acad. Sci. USA 81, 1008–1011.
[6] Haritos, A.A., Salvin, S.B., Blacher, R., Stein, S. and Horecker, B.L. (1985) Proc. Natl. Acad. Sci. USA 82, 1050–1053.
[7] Haritos, A.A., Blacker, R., Stein, S., Caldarella, J. and Horecker, B.L. (1985) Proc. Natl. Acad. Sci. USA 82, 343–346.
[8] Pan, L.-X., Haritos, A.A., Wideman, J., Komiyama, T., Chang, M., Stein, S., Salvin, S.B. and Horecker, B.L. (1986) Arch. Biochem. Biophys. 250, 197–201.
[9] Panneerselvan, C., Wellner, D. and Horecker, B.L. (1988) Arch. Biochem. Biophys. 265, 454–457.
[10] Eschenfeldt, W.H. and Berger, S.L. (1986) Proc. Natl. Acad. Sci. USA 83, 9403–9407.
[11] Goodall, G.J., Dominguez, F. and Horecker, B.L. (1986) Proc. Natl. Acad. Sci. USA 83, 8926–8928.
[12] Haritos, A.A., Tsolas, O. and Horecker, B.L. (1984) Proc. Natl. Acad. Sci. USA 81, 1391–1393.
[13] Gomez-Marquez, J. and Segade, F. (1988) FEBS Lett. 226, 217–219.
[14] Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529–539.
[15] Gurdon, J.B. (1976) J. Embryol. Exp. Morphol. 36, 523–540.
[16] Bonner, W.M. (1975) J. Cell Biol. 64, 421–430.
[17] Laemmli, U.K. (1970) Nature 277, 680–685.
[18] Laskey, R.A. and Mills, A.D. (1977) FEBS Lett. 82, 314–316.
[19] Dingwall, C. and Allan, J. (1984) EMBO J. 3, 1933–1937.
[20] Goldfarb, D.S., Garlepy, J., Schoolnik, G. and Kornberg, R.D. (1986) Nature 322, 641–644.
[21] Kalderon, D., Richardson, W.D., Markham, A.F. and Smith, A.E. (1984) Nature 311, 33–38.
[22] Abercrombie, B.D., Kneale, G.G., Crane-Robinson, C., Bradbury, E.M., Goodwin, G.H., Walker, J.M. and Johns, E.W. (1978) Eur. J. Biochem. 84, 173–177.
[23] Cary, P.D., King, D.S., Crane-Robinson, C., Bradbury, E.M., Rabbani, A., Goodwin, G.H. and Johns, E.W. (1980) Eur. J. Biochem. 112, 577–588.
[24] Cary, P.D., Turner, C.H., Mayes, E. and Crane-Robinson, C. (1983) Eur. J. Biochem. 131, 367–374.
[25] Carballo, M., Puigdomenech, P. and Palau, J. (1983) EMBO J. 2, 1759–1764.
[26] Dorbic, T. and Wittig, B. (1987) EMBO J. 6, 2393–2399.
[27] Beatriz Levy, W., Wong, N.C.W. and Dixon, G.H. (1977) Proc. Natl. Acad. Sci. USA 74, 2810–2814.
[28] Stoute, J.A. and Marzluff, W.F. (1982) Biochem. Biophys. Res. Commun. 107, 1279–1284.
[29] Tremethick, D.J. and Molloy, P.L. (1986) J. Biol. Chem. 261, 6986–6992.
[30] Lapeyre, B., Bourbon, H. and Amalric, F. (1987) Proc. Natl. Acad. Sci. USA 84, 1472–1476.
[31] Pentecost, B. and Dixon, G.H. (1984) Biosci. Rep. 4, 49–57.
[32] Orrick, L.R., Olson, M.O.J. and Busch, H. (1973) Proc. Natl. Acad. Sci. USA 70, 1316–1320.
[33] Ma, J. and Ptashne, M. (1987) Cell 48, 847–853.
[34] Hope, I.A. and Struhl, K. (1986) Cell 46, 885–894.
[35] Dingwall, C., Sharnick, S.V. and Laskey, R.A. (1982) Cell 30, 449–458.
[36] Dilworth, S.M., Black, S.J. and Laskey, R.A. (1987) Cell 51, 1009–1018.