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COMPARISON OF *IN VIVO* AND *IN VITRO* TRANSLATION OF THE *ANEMIA PHYLLITIDIS* POLY $(A)^+$ RNA DURING SPERMATOGENESIS[1]

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Abstract

Ribonucleic acid was extracted from 10 days old reproductive prothalli of the fern *Anemia phyllitidis*. Polyadenylated RNA was isolated by using poly (U)-sepharose chromatography and its composition was studied by translating the poly $(A)^+$ RNA *in vitro* in rabbit reticulocyte lysate. Electrophoretic analysis of the *in vitro* translation products and of proteins synthesised *in vitro* gave many common components, but there were also distinct quantitative and qualitative differences in the protein composition of the products of the two fractions. The *in vitro* synthesised proteins formed a heterogenous population, ranging in molecular weight from 10,000 to 72,000, while those from *in vivo* ranged from 10,000 to over 120,000 Daltons.

Introduction

The presence of poly $(A)^+$ sequences in messenger RNAs from plants and animals is now well established (Brawerman, 1981). The presence of polyadenylic sequences in the RNA of the vegetative prothalli of fern *Anemia phyllitidis* has been reported in an earlier study (Iqbal, 1977), and now its presence has also been shown in the dry spores of *Anemia* (Fechner, unpublished). Numerous eukaryotic cell-free systems efficiently translate messenger RNAs from viral or eukaryotic origin. With these systems a potentially useful tool for examining differences in mRNA populations has become available (Tobin & Klein, 1975). By characterising the translation products of mRNA, one can show differences in the relative content of different messengers, as well as, in the overall levels of mRNA and eventually one can look for more specific changes in mRNA than can be detected by simply measuring the poly $(A)^+$ content of RNA.

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In the present study we have compared the *in vivo* and *in vitro* translation products from 10 days old reproductive prothalli of *Anemia*. It should now also be possible to make comparisons between the mRNA component of *Anemia* in various developmental stages leading to antheridium formation and spermatogenesis.

Material and Methods

(i) *Growth conditions:* Spores of *A. phyllitidis* were sterilised (10% chlorox for 5 min.), rinsed thoroughly in distilled water and sown on the surface of a mineral salt medium (Mohr, 1956), alongwith gibberellic acid ($5x10^{-5}$ g/ml). In each Petri dish (ϕ10 cm) 20 ml of the nutrient medium and 10 mg dry weight of spores were added. Growth conditions were similar as reported earlier by Schraudolf (1966).

(ii) *In vivo labelling and extraction of proteins:* Spores were sown and grown under sterile conditions upto 10 days in mineral salt medium containing gibberellic acid ($5x 10^{-5}$ g/ml) and 2.0 μCi of a complete ^{14}C-amino acid mixture (specific activity 57 mCi /m atom carbon, Radiochemical Centre, Amersham) in a total of 20 ml of growth medium in conical flasks.

For extraction of soluble proteins the 10 days old reproductive prothalli were homogenised in 0.1 M Tris-HCl extraction buffer pH 7.6; + 5 mM mercaptoethanol (ratio of buffer to material 3:1). Following homogenisation the sample was centrifuged at 6000 rpm for 20 min. at 4°C. The resulting supernatant was then dialysed overnight at 4°C against 0.01 M Tris-HCl (pH 7.6), + 5 mM mercaptoethanol and proteins were precipitated with ice cold trichloroacetic acid (10% wt/vol). The precipitated proteins were washed 3-4 times with cold acetone and dried under stream of nitrogen. Dried proteins were dissolved in an electrophoresis sample buffer (10% SDS 30 ml, 0.5 M Tris-HCl 12.5 ml, pH 6.8; Beta mercaptoethanol 5.0 ml, water 42.5 ml, 10% glycerol 10.0 ml and 0.05% Bromophenol blue), sonically oscillated for complete dissolution. The protein content of the sample was determined by the method of Lowry *et al.* (1951), and adjusted to approximately 10-15 μg/10 μl for electrophoresis.

(iii) *Isolation of poly $(A)^+$ RNA:* Extraction of total RNA from unlabelled 10 days old reproductive prothalli was carried out as described earlier (Iqbal, 1977). Poly $(A)^+$ RNA was separated by affinity chromatography using poly (U)-sepharose column. Ethanol precipitated RNA was dissolved in binding buffer (10 mM Tris-HCl, 0.3 M NaCl, 0.1% SDS; pH 7.5) and passed twice over poly (U) sepharose column. Unbound RNA was washed with wash buffer (10 mM Tris-HCl, 0.1 M NaCl, 0.1% SDS; pH 7.5) until zero absorbance. The column was heated to 50°C for 20 min. and poly $(A)^+$ RNA was eluted with the elution buffer (10 mM Tris-HCl pH 7.5, 0.1% SDS). Different 1 ml fractions of the elute were pooled, dialysed and finally precipitated by addition of 2 volumes of ethanol alongwith 0.1 vol. of 20% potassium acetate (pH 5.5), overnight at –20°C.

Both ethanol precipitated total RNA and poly $(A)^+$ RNA were dried under stream of nitrogen, dissolved in bidistilled water and immediately used for *in vitro* translation.

(iv) *In vitro translation:* For *in vitro* translation, the reticulocyte lysate-amino acid, L–(^{3}H) was used (New England Nuclear Chemicals GmbH, Dreieich, West Germany). Protein synthesis was performed in a total incubation volume of 25 µl containing 13 µl of premix (specified by the supplier), 10 µl of endonuclease treated rabbit reticulocyte lysate and 2 µl of an aquous RNA solution. Generally the incubation mixture contained 5 µCi of translation grade L–(^{3}H)–leucine (2 mCi/ml; specific radioactivity 117 Ci/m mol). Total RNA was added at 2–5 µg per reaction mix. The exact amount of poly $(A)^+$ RNA was not known because of limitations of quantity but was of the order of 0.5–2 µg per reaction mix. Assay tubes were placed into the 37°C water bath to begin the incubation. After one hr. the incubation was stopped by removing the assay tubes from the incubation bath and placed on ice. From each assay tube 5 µl aliquots were added to 0.5 ml. of sodium hydroxide/hydrogen peroxide (N NaOH containing "40 vol" 5% H_2O_2), mixed and heated at 37°C for 10 min. to hydrolyse aminoacyl–tRNA and to decolourise the mixture. The tubes cooled in ice-bath and protein was precipitated by adding 3 ml of ice-cold 25% trichloroacetic acid containing 1 % (w/v) bovine albumin. Precipitated protein was filtered on Whatman GF/C filters, dried and counted in a liquid scintillation counter (Isocap/300 Liquid Scintillation Counter, Searle Nederland BV. Nuclear Chicago Division).

(v) *Protein assay:* The soluble protein content from *in vivo* and *in vitro* experiments was determined by the method of Lowry *et al.*, (1951). The volume of each sample necessary to give approximately 10–15 µg/µl of protein was adjusted for electrophoresis.

(vi) *Electrophoresis.* One dimensional polyacrylamide gel system of Laemmli (1970) was used. Approximately 10–15 µg amount of protein in 10 µl sample buffer was applied per slot. Electrophoresis was performed at a constant current of 12 mA with 12.5% polyacrylamide gels of 1 mm thickness for 8 hr. After electrophoresis the gels were prepared for fluorography as described by Bonner & Laskey (1974). Nuclear and cytoplasmic proteins of $Ad^{2+}ND_2$ non-disinfectant Hela cells were used as molecular weight markers, which was a kind gift from Dr. W. Deppert (Biochemistry Department, Ulm University).

Results

(i) *In vitro translation.* Total RNA and poly $(A)^+$ RNA isolated from 10 days old reproductive prothalli of *Anemia* are capable of initiating protein synthesis in rabbit reticulocyte lysate cell free system. Total RNA stimulated protein synthesis 4–7 fold over that of the endogenous activity of the reticulocyte lysate system. The stimulatory

activity of different poly $(A)^+$ RNA preparation was variable (8–40 times stimulation). The maximum value obtained was about 40 fold (Table 1).

Table 1. Translation of total RNA and poly $(A)^+$ RNA from 10 days old reproductive prothalli of *Anemia phyllitidis*.

	^{3}H-incorporation (cpm)+	
Addition	Experiment I	Experiment II
No RNA	2484	2153
Total RNA	17165	13487
Poly $(A)^+$ RNA	78297	85742

+ = Data from two representative experiments. In all 37 translations were run in *in vitro*.

(ii) *Analysis of in vitro translation products.* Translational products from total RNA and poly $(RNA)^+$ were characterised by electrophoresis on one-dimensional poly acrylamide slab gels. The predominant protein products approximately ranged in molecular weight of 10,000–72,000 Daltons, although minor components of high molecular weight were also detected in some over exposed fluorograms. At least 35 distinct separate bands can be seen both in total RNA (Fig. 1, Lane 2), and poly $(A)^+$ RNA (Fig. 1, Lane 3) directed translational products. The protein composition of repeated translation product of total and poly $(A)^+$ RNA preparations was constant. This suggests that the protein composition was representative of the relative concentration of the individual mRNA species at this particular stage of development. Qualitative differences were found in the translation products of total RNA and poly $(A)^+$ RNA (Fig. 1; differences marked by arrows). Two to three bands were also detected even in control system in the absence of added RNA, as the endogenous products of the translation system (Fig. 1, Lane 1).

(iii) *Analysis of in vivo translation products.* The composition of proteins synthesised *in vivo* of 10 days old reproductive prothalli labelled with ^{14}C-amino acid mixture are shown in Fig. 1, Lane 4, alongwith the *in vitro* products (Fig. 1, Lane 2,3). The predominant protein products ranged in molecular weight from 10,000 to 120,000 Daltons, a much wider range when compared to the *in vitro* products. Approximately 50 distinct protein bands are visualised from the fluorogram, a number far greater than the *in vitro* products (approximately 35 bands). However, in molecular range of 10,000–72,000, where almost all *in vitro* synthesised proteins are confined, the *in vivo* protein products closely resembled those from *in vitro* translation products, although distinct quantitative differences and on keen analysis a small number of qualitative differences could be observed (Fig. 1; differences marked with arrows on right hand side of lane 4).

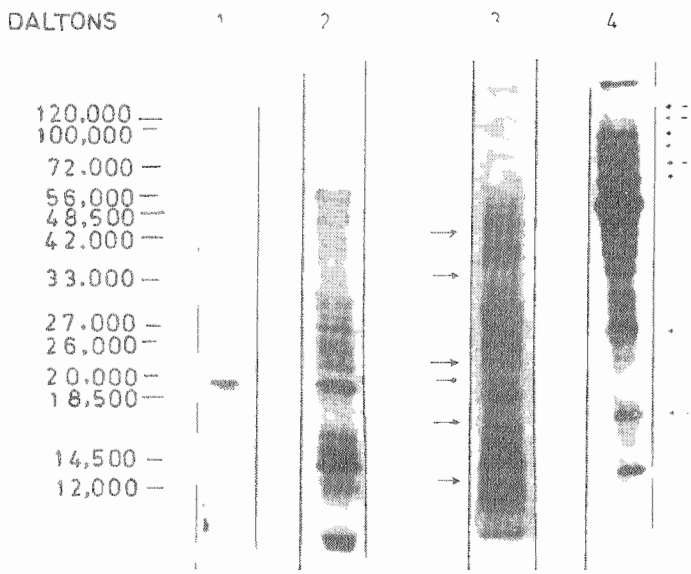

Fig. 1 Fluorogram of *in vivo* and *in vitro* synthesized proteins from 10 days old reproductive prothalli of *Anemia phyllitidis*, analysed by one-dimensional SDS-polyacrylamide gels (12.5%)

Lane 1: Endogenous incorporation of (^{3}H)-leucine into proteins by the unstimulated reticulocyte lysate system.

Lane 2 Radioactive translation products from an *in vitro* reaction stimulated by total RNA.

Lane 3 Radioactive translation products from an *in vitro* reaction stimulated by poly $(A)^+$ RNA

Lane 4 *In vivo* incorporation of label (^{14}C-amino-acid mixture) into proteins.

The number on the left are molecular weights of marker proteins

(i) Differences between *in vitro* translation products of total RNA and poly $(A)^+$ RNA are marked by arrows between the lane 2 and 3.

(ii) Differences between *in vitro* and *in vivo* translation products are marked by arrows on right hand side of lane 4.

Discussion

Although the *in vitro* synthesised protein band pattern closely resembled those from *in vivo* synthesised proteins, some clear cut differences were observed. For example, the absence of high molecular weight proteins from *in vitro* translations (over 72,000 Daltons) and the lack of perfect correlation in the protein bands made *in vitro* in the rabbit reticulocyte-lysate system and those of synthesised *in vivo*. The absence of high molecular weight proteins from the translation product of poly $(A)^+$ RNA probably reflects the loss of large poly $(A)^+$ RNA molecules during poly (U)-sepharose chromatography, a phenomenon which has also been reported by Hall & Lim (1981) in rat. The discrepancies observed in the protein bands from *in vivo* and *in vitro* systems in *Anemia* has also been observed with mRNAs translational products in *Xenopus* (Darnbrough & Ford, 1976), wheat embryos (Cuming & Lane, 1978) and in pollen grains of *Tradescantia* (Frankis & Mascarenhas, 1980). Various reasons for this lack of correlation has

been suggested, e.g. the *in vitro* initiation and/or termination sites for protein synthesis might differ from those used *in vivo;* proteins synthesised *in vivo* might undergo post-translational modifications which do not take place in the cell free system, etc. (Both *et al.,* 1975; Prives *et al., 1974; Roberts et al.,* 1973).

Despite the limitations outlined above, total RNA and poly $(A)^+$ RNA from 10 days old reproductive prothalli of *Anemia* have been shown to initiate and synthesize efficiently a number of proteins *in vitro.* The next step in this work might be an attempt to positively identify one or more of these translation products by using specific antisera and to try to relate changes in the levels of their mRNA to changes in the protein production *in vivo.* This should facilitate a study of cell regulatory mechanism that control transcription in the developmental course of antheridium initiation and spermatogenesis.

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