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Staining of ribonucleic acid in the oesophageal bulb of *Xiphinema index* by bromination and microwave immunohistochemistry

Jenifer M. ROBB and John M. S. FORREST

Zoology Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland.

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Summary — The specific staining of RNA in the oesophageal bulbs of *Xiphinema index* is described. Sections of excised oesophageal bulbs embedded in methacrylate or " LR White " resin were brominated and treated with a monoclonal antibody against 5-bromodeoxyuridine. Staining was achieved using a secondary antibody with immunogold silver enhancement. Time required for incubation steps was substantially reduced by using microwave irradiation.

Résumé — ***Coloration de l'ARN dans le bulbe oesophagien de* Xiphinema index *par immunochimie et par utilisation du brome et d'un four à micro-ondes*** — La coloration spécifique de l'ARN dans le bulbe oesophagien de *Xiphinema index* est décrite. Des coupes de bulbes oesophagiens pris dans une résine au méthacrylate ou au " LR white " ont été traitées au brome puis par un anticorps monoclonal de souris anti-5-bromodeoxyuridine. L'ARN se colore à l'aide d'un anticorps de chèvre anti-souris conjugué à de l'or colloïdal et à des sels d'argent qui intensifient la coloration. Le temps d'incubation est fortement réduit par l'utilisation d'un four à micro-ondes.

Key-words : RNA, staining, *Xiphinema*.

Previous work on the nature of secretions from plant-parasitic nematodes indicated the presence of nucleic acids within the nematode salivary glands (Cardin & Dalmasso, 1985; Sundermann & Hussey, 1988). However, as yet little is known regarding the type of nucleic acid, or its distribution within the gland.

A number of histochemical techniques are available for localising RNA in fixed sections (Pearse, 1982), but in most of these procedures, DNA is also stained, preventing the specific recognition of RNA. In this report an immunohistochemical method is described which enables the specific localisation of the uracil base which is present in RNA only. Under certain conditions, the uracil base can be brominated and subsequently recognised by the monoclonal antibody, Mo-Bu-1 (Harms *et al.*, 1986; Harms & Hardonk, 1989). DAPI (4,6-diamidino-2-phenylindole) which stains DNA but not RNA is used to confirm the specificity of the reaction.

Materials and methods

Oesophageal bulbs from *Xiphinema index* were excised into 0.1 % glutaraldehyde, 3 % paraformaldehyde for 2 h at room temperature. Care was taken to select specimens which had appeared to have fed recently i.e. with a darkened, turgid intestine. The bulbs could be excised cleanly by cutting through the body immediately behind the bulb; the difference in osmotic pressure caused the bulb to be extruded. After thorough washing in distilled water clusters of five to ten bulbs were blocked in 1 % water agar and dehydrated through an alcohol series — 60, 70, 80, 90, 100, 100 % — for 10 min each prior to methacrylate infiltration or 50, 60, 70, 70 % prior to London resin infiltration.

Infiltration with methacrylate resin (80 % butyl, 20 % methyl methacrylate) took place on a rotator at 4 °C. Agar blocks were infiltrated in the following resin combinations for 1 h each : 30 % resin, 70 % absolute alcohol; 50 % resin, 50 % absolute alcohol; 70 % resin, 30 % absolute alcohol; 100 % resin. Finally blocks were polymerised overnight under long wave ultra-violet excitation in fresh resin containing 0.05 % benzoin ethyl ether (Sigma Ltd, Poole, Dorset). This procedure was carried out at room temperature in the absence of oxygen. Infiltration with " LR White " (a blend of acrylic monomers) resin, took place on a rotator at 4 °C. Agar blocks were infiltrated with resin overnight prior to polymerisation in fresh resin at 50 °C for 36 h. For light microscopy, sections 1 µm thick, were cut on an LKB microtome and dried on to multiwell slides coated in 3-aminopropyltriethoxysilane (Sigma Ltd) (Henderson, 1989).

To reduce autofluorescence and cut down background binding to free aldehyde groups, sections were incubated at room temperature in 0.1 % sodium borohydride in PBS (0.1 M, pH 7.1) for 10 min (Chayen *et al.*, 1973) then rinsed in distilled water.

To test the specificity of the staining method for RNA, sections were incubated for 1 h at room tempera-

ture with ribonuclease type A (Sigma Ltd) at 1 mg/ml distilled water. Sections were rinsed in distilled water, brominated and stained as described below.

To confirm that DNA is not stained sections were treated for 1 h at room temperature with deoxyribonuclease I, ribonuclease free (Sigma Ltd), 0.1 mg/ml in 50 mM Tris-HCl, pH 7.4 containing 3 mM $MgCl_2$ and treated as described below. Control sections were incubated in 50 mM Tris-HCl, pH 7.4 containing 3 mM $MgCl_2$ for 1 h at room temperature.

4,6-diamidino-2-phenylindole (DAPI) binds to A-T rich regions of double stranded DNA. It is not absolutely specific to DNA as it shows affinity for some phenolic compounds and microtubules (James & Jope, 1978; Coleman & Goff, 1985). Sections were incubated at room temperature in the dark for 1 h in 0.1 µg/ml DAPI (Sigma Ltd) in distilled water.

The uracil residues of RNA were brominated as follows : Sections were treated for 30 min at room temperature in 0.0025 % w/v bromine in 0.2 % potassium bromide (Harms & Hardonk, 1989) followed by washing : five times for 2 min in distilled water; 5 min in 0.5 % sodium bisulphite; 5 min in distilled water; and finally three 2 min rinses in PBS 0.1 M, pH 7.4.

To block non-specific binding of the monoclonal antibody sections were first treated with PBS 0.1 M, pH 7.2 containing 0.1 % BSA (Sigma Ltd) and 0.4 % gelatin (20 % stock, EM grade, Janssen N. V. Olen, Belgium) 10 min followed by a 3 min rinse in PBS containing 0.1 % BSA. To label the brominated RNA sections were incubated with undiluted Mo-Bu-1 antiserum specific for 5-bromodeoxyuridine (Harms *et al.*, 1986) plus 0.1 % BSA for 5 min in the microwave at 42 % with a 200 ml water load (Van de Kant *et al.*, 1980). After rinsing twice for 3 min in PBS, sections were blocked in PBS 0.1 M pH 7.2 containing 0.1 % BSA and rinsed once more, prior to incubation with Goat anti Mouse (GAM) auroprobe one (Janssen N. V.), diluted 1/50 in PBS plus 0.1 % BSA. Incubation was again for 5 min in the microwave, at 42 % with a 200 ml water load after which sections were washed in PBS three times for 10 min, followed by distilled water for 10 min.

Silver enhancement of gold labelled sections was carried out using Intense EM Silver (Janssen N. V.). Sections were then rinsed in distilled water and mounted in 50 % glycerol. They were viewed on an Olympus BH 2-RFL fluorescence microscope in bright field illumination or epifluorescence. Photographs of sections were taken using × 100 oil immersion lens on TMAX 100 film (Kodak Ltd, Ilford, Essex).

Results

The staining procedure is summarised in Table 1. This produces well labelled sections with very little or no background (Fig. 1 A, C). Bromination for longer than 30 min (up to 2 h) did not alter the staining pattern. Any reduction in incubation time below 30 min did, however, result in a loss of staining intensity. Sections incubated with RNase showed an almost total loss of staining with Mo-Bu-1 compared to untreated sections (Fig. 1 D). When the incubation period with the enzyme was increased to 2 h, loss of staining was complete (Fig. 1 B). In contrast, sections receiving prior treatment with DNase stained with similar intensity to those incubated in buffer only (Fig. 2). In some cases there was a very slight reduction in staining intensity, thought to be due to minute amounts of RNases present in the buffer solutions. DAPI stained the sections brightly, producing a kind of halo effect (Fig. 3 A). In addition RNase treated sections also stained brightly with DAPI, showing DNA was still present, so confirming that loss of staining was due solely to the absence of RNA. DNase treated sections which showed Mo-Bu-1 staining did not subsequently stain with DAPI (Fig. 3 B).

Table 1. Timescale of immunostaining using microwave irradiation.

Sodium borohydride	10 min
Rinse (dH_2O)	3 min
Bromination	30 min
Rinse (dH_2O)	5 × 2 min
(sodium bisulphite)	5 min
(dH_2O)	5 min
(PBS)	3 × 2 min
Block	10 min
Rinse	3 min
Mo-Bu-1	5 min
Rinse	3 min
Block	10 min
Rinse	3 min
GAM auroprobe 1	5 min
Rinse (PBS)	3 × 10 min
(dH_2O)	10 min
Silver enhancement	10 min
	Total = 2 h 38 min
	Total antibody incubations = 10 min

Discussion

In this report, a method to demonstrate the presence of RNA in metacrylate and London resin sections of *X. index* oesophageal bulbs is detailed.

In aqueous solution at room temperature, only the uracil base is brominated to form 5-bromouracil (Kanngiesser, 1959) which is subsequently recognised by the Mo-Bu-1 antibody (Harms & Hardonk, 1989). The specificity of the staining method for RNA alone is supported by its sensitivity to RNase as opposed to DNase pretreatment, its dependence on the bromination procedure and the specificity of the monoclonal antibody involved (Harms *et al.*, 1986).

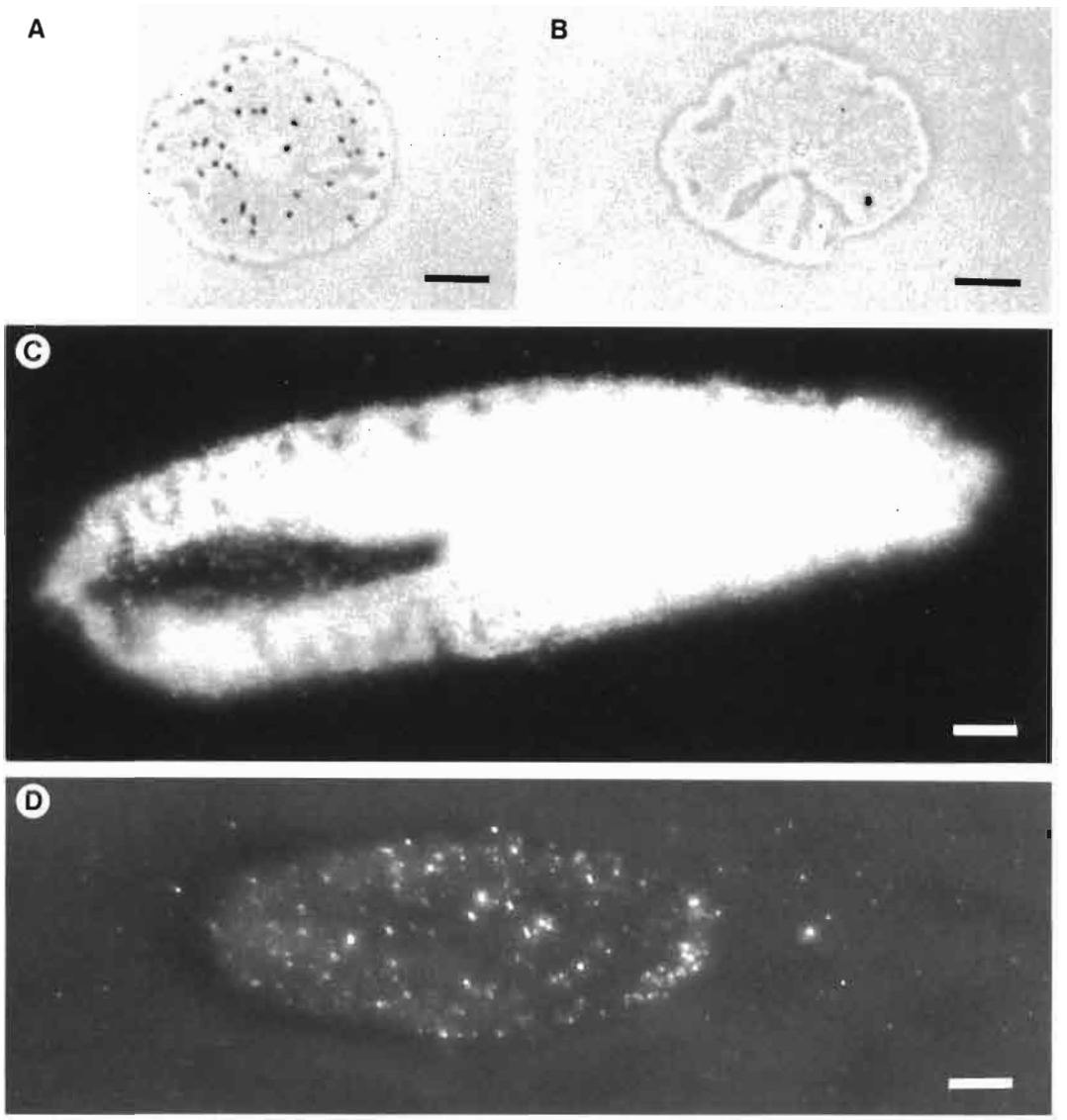

Fig. 1. Transverse section of oesophageal bulb of *Xiphinema index* after bromination and labelling with Mo-Bu-1/IGSS under bright field. — A : After bromination and labelling with Mo-Bu-1/IGSS; B : After treatment with RNase for 2 h prior to bromination and labelling with Mo-Bu-1/IGSS; Oblique section of oesophageal bulb viewed under epifluorescence; C : After bromination and labelling with Mo-Bu-1/IGSS; D : After treatment with RNase for 2 h prior to bromination and labelling with Mo-Bu-1/IGSS *(Scale bar = 10 µm).*

The staining obtained at light microscope level was enhanced using the IGSS method. This resulted in localised specific staining, against a clear background and as an added advantage, permanence of staining. The use of microwave irradiation in conjunction with IGSS is a relatively new development, the first reports being by Jackson *et al.* (1988), Van de Kant *et al.* (1988) and Boon *et al.* (1989). However, results to date have been very promising with new applications continually being found. In immunostaining, the aim is to attach the maximum amount of label with the minimum of non-specific background staining. To obtain a reaction, reagents are required first to diffuse into the tissues, then, to prevent background, unbound reagent must diffuse out. Microwave irradiation speeds up these diffusion and incubation steps which in turn lead to a reduction of background label. In the method described here, incubation times are reduced by over 85 %. Very

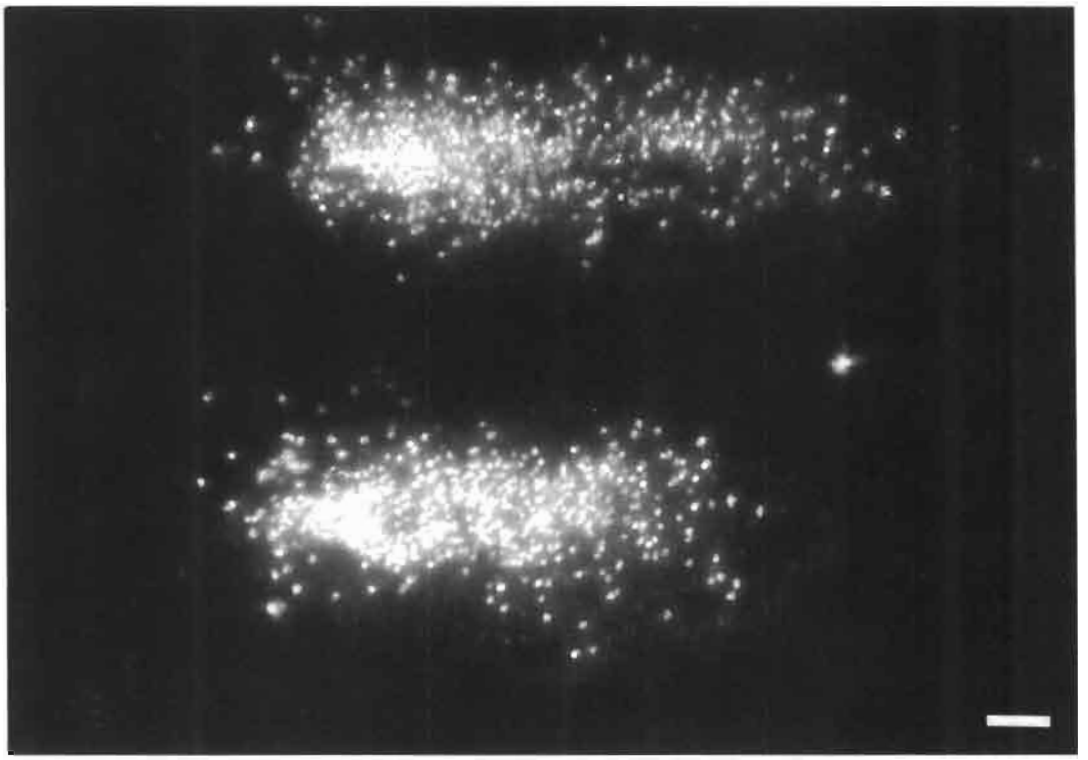

Fig. 2. Oblique sections of oesophageal bulbs of *Xiphinema index* viewed under epifluorescence after treatment with DNase prior to bromination and labelling with Mo-Bu-1/IGSS *(Scale bar = 10 µm).*

little can be said about the exact location or type of RNA within the bulb. Staining was not uniform throughout the bulb, and in oblique sections a striated pattern was often seen. This is perhaps surprising, because RNA is not cell specific, and so the stain might be expected to illuminate the whole structure. Perhaps different RNA species are not equally accessible. Clearly, there is a possibility that this technique may be used to localise RNA to oesophageal bulb glands and/or ducts, thus suggesting active secretion. This can only be achieved by investigation at electron microscope level.

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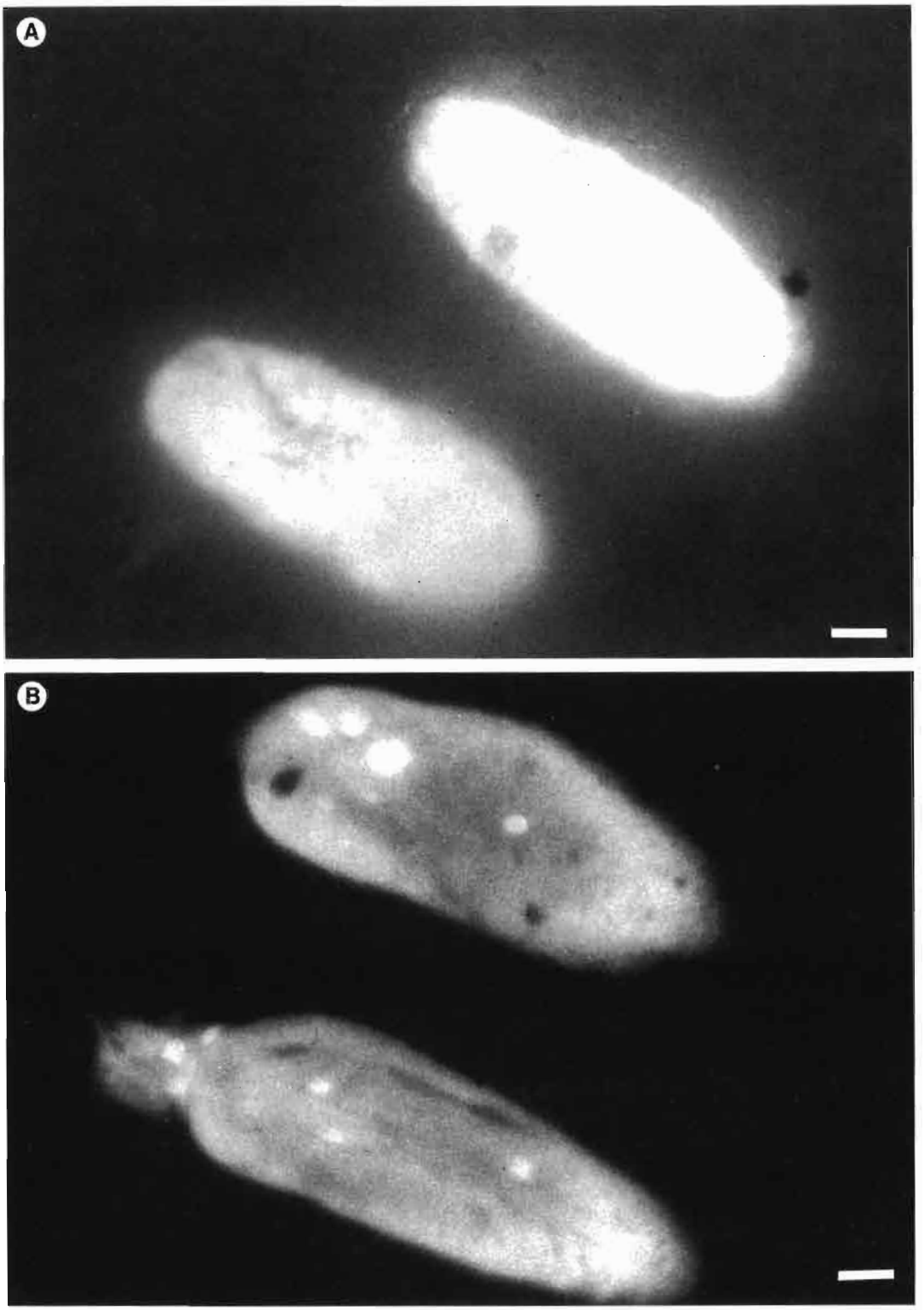

Fig. 3. Oblique sections of the oesophageal bulbs of *Xiphinema index* viewed under UV excitation. A : After incubation with DAPI; B : After treatment with DNase for 2 h, bromination and labelling with both Mo-Bu-1/IGSS and DAPI *(Scale bars = 10 µm).*

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