An assessment of some methodological criticisms of studies of RNA efflux from isolated nuclei

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RNA efflux from isolated nuclei can be studied either as a means of elucidating the general mechanism of nucleo-cytoplasmic RNA transport, or as part of an investigation of the processing and utilization of particular gene transcripts. The present paper describes an assessment of three methodological criticisms of RNA-efflux measurements that are made for the former reason: for such measurements, it is sufficient to show that the post-incubation supernatant RNA is similar overall to homologous cytoplasmic mRNA, rather than to nuclear RNA, that is nevertheless of intranuclear origin, and that alterations to the medium during experiments do not markedly perturb this general nuclear restriction. The results seem to justify the following conclusions. (1) Although degradation of the nuclear RNA occurs during incubation *in vitro*, this process does not account for the appearance of RNA in the postnuclear supernatant. The degradation can be largely prevented by the addition of serine-proteinase inhibitors without altering the RNA efflux rate. (2) Some adsorption of labelled cytoplasmic RNA to the nuclear surface occurs during both isolation and incubation of the nuclei, and some desorption occurs during incubation. However, these effects introduce errors of less than 10% into the measurements of efflux rates. (3) Exogenous acidic polymers, including polyribonucleotides, disrupt nuclei and increase the apparent RNA efflux rate by causing leakage of nuclear contents. However, this effect can largely be overcome by including the nuclear stabilizers spermidine, Ca^{2+} and Mn^{2+} in the medium. In terms of this assessment, it appears that RNA efflux from isolated nuclei in media containing nuclear stabilizers serves as a reasonable model for transport *in vivo*.

RNA efflux measurements may be made (a) as part of a study of the general mechanism of nucleo-cytoplasmic RNA transport or (b) in the investigation of the processing and availability for translation of the transcripts of particular genes. These two purposes entail slightly different methods and therefore slightly different sets of methodological criteria. For the former purpose, measurements of the rate and final extent of total RNA efflux is sufficient, provided that it can be demonstrated that most of the supernatant RNA resembles cytoplasmic rather than nuclear material. For the latter, any contamination of the mRNA of interest with nuclear precursors is a serious problem: average rates and extents of RNA efflux are not

important measurements here. An important study by Jacobs & Birnie (1982) addressed this latter problem. Using complementary-DNA probes, they showed that, when liver and hepatoma nuclei were incubated in a medium similar to those used by Yu *et al.* (1972) and Agutter & McCaldin (1979), contamination of the eluted mRNA with nuclear precursors might be a significant problem only with regard to low-abundance species. The present paper addresses the former problem, and, despite the different criteria adopted, the conclusions about the suitability of the medium used are compatible with those of Jacobs & Birnie (1982).

With regard to mechanistic as opposed to RNA-processing studies, several general methodological criticisms can already be answered. For instance, when the media of Yu *et al.* (1972) or Agutter & McCaldin (1979) are used, the RNA left in the post-nuclear supernatant after incubation

Abbreviations used: NTPase, nucleoside triphosphatase (EC 3.6.1.15); $\text{p}[\text{CH}_2]\text{ppA}$, adenosine 5'-[β,γ -methylene]triphosphate.

resembles homologous cytoplasmic mRNA according to competition-hybridization evidence (Schumm & Webb, 1974; Agutter, 1982). When the RNA in the post-nuclear supernatant is translated *in vitro*, normally occurring tissue polypeptides are formed (Palayoor *et al.*, 1981). Perturbations of the nucleocytoplasmic RNA distribution such as occur during hepatocarcinogenesis (Shearer, 1974) are reflected in corresponding alterations in RNA efflux from isolated nuclei (Schumm *et al.*, 1977; Clawson *et al.*, 1980b). The supernatant RNA certainly cannot have leaked from a small number of damaged nuclei, because Feldherr (1980) showed that physical damage to nuclei *in situ* did not alter nucleocytoplasmic RNA distributions, and Agutter & Suckling (1982b) showed that high concentrations of colchicine, which caused constriction of the pore-complexes while disordering the lipid bilayers of the envelope, completely inhibited RNA efflux from nuclei *in vitro* irrespective of mechanical damage. The proteins eluted along with the RNA resemble neither those associated with heterogeneous nuclear RNA nor any other major class of nuclear proteins (Agutter, 1982). Finally, when a short prelabelling time is used before rats are killed, there is a delay, which the authors attributed to the completion of processing of the labelled mRNA precursors, before efflux of labelled RNA from liver nuclei *in vitro* begins (Moffett & Webb, 1981).

Nevertheless, important criticisms remain. Among these are the following.

(1) It is possible that at least some of the observed RNA efflux results from degradation of nuclear contents, e.g. by proteinases or ribonucleases, and therefore does not represent a physiological transport process.

(2) The labelled RNA in the post-nuclear supernatant may not be a true measure of the amount of labelled RNA leaving the nucleus. Some RNA might leave the nucleus and be absorbed by the outer membrane. Conversely, adsorbed labelled material may be eluted from the nuclear surface during incubation.

(3) High concentrations of total yeast RNA are frequently added to incubation media as a carrier. Some workers have reported that this increases the rate of RNA efflux (Sato *et al.*, 1977; Ishikawa *et al.*, 1978), and in one study some exogenous RNA species, notably poly(U), seemed to cause almost total lysis of the nuclei (Goidl *et al.*, 1975).

The aim of the present paper is to examine the validity of these criticisms as applied to variants of two of the incubation media described in the literature. The results suggest: that the first criticism, although it cannot be dismissed, does not obviously cast doubts on the literature in the field; that the second is essentially invalid; and that the validity of the third varies with the medium used.

Materials and methods

ATP (disodium salt, grade II), colchicine, heparin (sodium salt, 170 USP units/mg), *N*-ethylmaleimide, phenylmethanesulphonyl fluoride, yeast RNA and poly(U) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Phenylmethanesulphonyl chloride was obtained from BDH, Poole, Dorset, U.K. Deoxyribonuclease I was obtained from Boehringer, Mannheim, West Germany, and [5-³H]-uridine was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents used were of AnalaR grade from BDH.

Male albino rats (100–150 g body wt.) were used throughout. For RNA-efflux experiments, they were injected with [5-³H]uridine (1 μ Ci/g body wt.) 45 min before they were killed.

Isolation of nuclei and nuclear envelopes

Nuclei were isolated by the method of Blobel & Potter (1966) and nuclear envelopes by a slight modification (Agutter & Gleed, 1980) of the method of Harris & Milne (1974).

Cytoplasmic ribonuclease inhibitor

The post-nuclear supernatant of the liver homogenate was centrifuged at 100 000 *g* for 1 h, and the cytoplasmic ribonuclease inhibitor was purified from the final supernatant by the method of Gagnon & de Lamirande (1973).

RNA-efflux measurements

Nuclei from [5-³H]uridine-prelabelled livers were incubated (5×10^6 nuclei/ml) in one of the media described below for 10 min at 30°C. The nuclei were pelleted by centrifuging at 2000 *g* for 10 min and trichloroacetic acid was added to the supernatants to a final concentration of 10% (w/v). The precipitate was recovered by centrifugation (2000 *g*, 15 min) and dissolved in 200 μ l of 0.1 M-NaOH, and radioactivity was counted as described by Agutter & McCaldin (1979). For analysis of molecular size distribution, the RNA was purified from the supernatant after incubation by the phenol/chloroform method of Brawerman (1974) and centrifuged in 5 ml tubes on linear 10–30% (w/v) sucrose gradients in 10 mM-acetate buffer, pH 5.2, over a 50% (w/v) sucrose cushion, as described by Schumm *et al.* (1973). The developed gradients were divided into 20 0.25 ml fractions, and radioactivity was counted in each fraction as described above. Total nuclear RNA was purified as described by Yannarell *et al.* (1977) and was also analysed on 10–30% sucrose density gradients.

The incubation media were as follows.

Medium A; 0.25 M-D-glucose, 10 mM-Tris/HCl buffer, 25 mM-KCl, 5.0 mM-spermidine, 5.0 mM-2-mercaptoethanol, 2.0 mM-MgCl₂, 0.5 mM-CaCl₂,

0.3 mM-MnCl₂, pH 7.6 (Agutter & McCaldin, 1979). In some experiments, the spermidine or the Ca²⁺ and Mn²⁺ were omitted.

Medium B: 0.25 M-sucrose, 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 10 mM-KCl, 2.5 mM-MgCl₂, 2 mM-dithiothreitol, pH 7.6 (Kletzein, 1980).

These media were supplemented with cytoplasmic ribonuclease inhibitor, yeast RNA, poly(U) and heparin as described in the Results section. Either 2.0 mM-ATP or 2.0 mM-EDTA was added to all incubations. When inhibitors of proteolysis were used, they were added to the media along with the nuclei and preincubated at 0°C for 10 min before ATP or EDTA was added and the incubation at 30°C started.

Chemical determinations and enzyme assays

Protein was determined by a modification (Maddy & Spooner, 1970) of the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. NTPase activity in nuclear envelopes was measured as described by Agutter *et al.* (1977).

Results

The size distribution of labelled RNA released into medium A during incubation depended on the presence of ATP in the medium, but was independent of the proteolytic inhibitors phenylmethanesulphonyl chloride and *N*-ethylmaleimide (Fig. 1*a*). In the absence of ATP, a small 4S peak was observed; in the presence of ATP, a broad peak around 10–12S predominated. These results are consistent with previous observations (Schumm & Webb, 1978; Agutter & McCaldin, 1979; Agutter, 1982). There was evidence for degradation of the nuclear RNA during incubation, and this appeared to be partially prevented by inhibitors of proteolysis (Fig. 1*b*). However, (i) even in the absence of proteolytic inhibitors, the labelled RNA remaining in the nucleus after incubation did not correspond in size distribution to that in the supernatant, and (ii) neither *N*-ethylmaleimide nor phenylmethanesulphonyl chloride inhibited RNA efflux overall (Table 1). The inhibition of efflux by phenylmethanesulphonyl fluoride seems to correspond to the inhibition of the NTPase by the same reagent (Table 1), in accordance with the well-established hypothesis that the NTPase supplies the energy required for RNA efflux and transport (Agutter *et al.*, 1976, 1979; Clawson *et al.*, 1978, 1980*a*; Murty *et al.*, 1980). Moreover, colchicine, which has been shown to inhibit ATP-dependent RNA efflux (Agutter & Suckling, 1982*b*), does so in spite of degradation of the nuclear material (Fig. 1). The implication of these results is that degradation of the nuclear RNA can occur during incubations, but is not responsible for the appearance of the labelled RNA in the supernatant.

Fig. 1. Effects of incubation conditions on size distributions of nuclear and supernatant RNA

Sucrose-density-gradient profiles of RNA in (a) post-nuclear supernatant after incubation and (b) nuclear pellet. The following incubation conditions were used: ○, EDTA; ●, ATP; ■, ATP + 5 mM-*N*-ethylmaleimide; ▲, ATP + 1 mM-phenylmethanesulphonyl chloride; □, ATP + 5 mM-colchicine. In Fig. 1(*b*), RNA from unincubated nuclei was run as a control (△). For standards, 28S and 18S rRNA and mixed tRNA were used. Results are means of three experiments each done in duplicate. Total nuclear radioactivity was $1.1 \pm 0.2 (\times 10^5)$ d.p.m./10⁷ nuclei.

The following experiments were performed to determine the role of adsorption and desorption of labelled RNA to and from the nuclear surface on the

Table 1. *Effects of inhibitors of proteolysis on RNA efflux and on NTPase activity*

Nuclei or nuclear envelopes were preincubated for 10 min with the inhibitors shown below. The first two inhibitors were added from a 100 mM stock in anhydrous propan-2-ol, and the solvent control involved adding propan-2-ol alone to the same final concentration (1%, v/v). After preincubation, RNA-efflux rate (nuclei) and NTPase activity (nuclear envelopes) were measured as described in the Materials and methods section, by using medium A supplemented with 300 μ g of yeast RNA/ml and cytoplasmic ribonuclease inhibitor for the efflux measurements. ATP-independent efflux was measured as the acid-precipitable label in the supernatant after incubation with EDTA, and ATP-dependent efflux was the acid-precipitable label in the supernatant after incubation with ATP minus ATP-independent efflux. Efflux was expressed as percentage of total nuclear radioactivity ($1.2 \times 10^4 \pm 0.4 \times 10^4$ d.p.m./ 10^7 nuclei) in the supernatants. All results are means $\pm$ S.E.M. for three experiments each performed in triplicate.

Inhibitor	RNA efflux		NTPase activity (μ mol of ATP/h per mg of protein)
	ATP-independent	ATP-dependent	
None	1.1 ± 0.2	2.1 ± 0.3	12.6 ± 1.2
Solvent control	1.3 ± 0.1	2.2 ± 0.2	12.0 ± 0.8
<i>N</i> -Ethylmaleimide (5 mM)	0.9 ± 0.2	2.1 ± 0	12.1 ± 0.5
Phenylmethanesulphonyl chloride (1 mM)	1.2 ± 0.3	1.7 ± 0.3	11.9 ± 0.7
Phenylmethanesulphonyl fluoride (1 mM)	1.1 ± 0.1	1.1 ± 0.2	7.8 ± 0.4

Table 2. *Effects of adsorption and desorption of RNA on measured efflux rates*

Experiments were performed on prelabelled nuclei and on unlabelled nuclei that had been mixed with the 800 g supernatant from prelabelled liver before purification. Incubations were performed in medium A, supplemented with 300 μ g of yeast RNA/ml and cytoplasmic ribonuclease inhibitor, in the presence of ATP or EDTA. Samples of nuclei were extracted with Triton X-100 before and after incubation, and the radioactivity in the Triton washes was counted. All values are expressed as d.p.m./ 10^7 nuclei, and are the means $\pm$ S.E.M. for three experiments each performed in triplicate.

Nuclei	Incubation conditions	$10^{-5} \times$	$10^{-3} \times$ Radioactivity	$10^{-2} \times$ Radioactivity in Triton washes	
		Total nuclear radioactivity	in post-nuclear supernatant after incubation	Before incubation	After incubation
Labelled	EDTA	1.2 ± 0.4	1.4 ± 0.3	4.8 ± 0.3	3.3 ± 0.2
	ATP		3.8 ± 0.4		3.0 ± 0.2
Unlabelled	EDTA	$4.5 \pm 0.5 (\times 10^{-3})$	0.16 ± 0.04	3.9 ± 0.2	2.4 ± 0.1
	ATP		0.18 ± 0.02		2.5 ± 0.6
Labelled, Triton-extracted	EDTA	1.0 ± 0.1	2.4 ± 0	—	—
	ATP		4.6 ± 0.5		

observed efflux rates. First, a [5- 3 H]uridine-prelabelled rat liver and an unlabelled rat liver were separately homogenized and centrifuged at 800 g for 10 min. The pellet from the unlabelled liver was mixed with the supernatant from the labelled liver and incubated for 10 min at 5°C; the nuclei were then purified by the method of Blobel & Potter (1966) and radioactivity was counted. Second, both these nuclei and the prelabelled nuclei were washed with 10 vol. of 2.0% (v/v) Triton X-100 in homogenization buffer (10 mM-Tris/HCl, 2 mM-MgCl₂, 250 mM-sucrose, pH 7.4) to remove the nuclear membranes together with adventitiously associated material (cf. Aaronson & Blobel, 1975), both before and after incubation in medium A, and radioactivity was counted in both supernatants and pellets. The results (Table 2) suggest that, although absorption and desorption of labelled material to and from the nuclear surface occurred, these processes were

responsible for errors of less than 10% of the radioactivity measured in the supernatant.

The effects of exogenous yeast RNA, poly(U) and heparin on RNA-efflux rates in the various media used are summarized in Table 3. The implication of the results is that all three acidic polymers increase the extent of efflux in the media that were used, but the effect is marked only when spermidine is omitted, and is barely significant if spermidine, Ca²⁺ and Mn²⁺ are all present. After a prolonged (30 min) incubation in the presence of heparin and of proteolytic inhibitors, the supernatant RNA resembled nuclear RNA in size distribution in medium B, but not in medium A (Fig. 2). These findings suggest that, although exogenous acidic polymers cause random elution of the RNA from isolated nuclei during incubation, this effect is largely offset by the bivalent cations that have previously been found to stabilize nuclei (Yu *et al.*, 1972; Agutter, 1980).

Table 3. *Effect of acidic polymers on RNA efflux rate*

Media A and B were modified as noted in the second column of the Table. Cytoplasmic ribonuclease inhibitor was present except where noted; the concentrations of spermidine, Ca^{2+} and Mn^{2+} were 5.0mM, 0.5mM and 0.3mM respectively. ATP-dependent and ATP-independent RNA efflux, expressed as a percentage of total nuclear radioactivity, were measured as described in the legend to Table 1. Results are means $\pm$ S.E.M. for three experiments, each performed in triplicate.

Medium	Modifications	Acidic polymer	Concn. ($\mu\text{g}/\text{ml}$)	RNA efflux	
				ATP-independent	ATP-dependent
A	—	—	—	1.0 ± 0.1	2.1 ± 0.2
A	—	Heparin	33	1.4 ± 0.3	2.3 ± 0.1
A	—	Yeast RNA	300	1.1 ± 0.3	2.4 ± 0
A	No ribonuclease inhibitor	—	—	< 0.1	0.3 ± 0.2
A	No ribonuclease inhibitor	Yeast RNA	300	0.9 ± 0.2	2.0 ± 0.2
B	—	—	—	2.6 ± 0.3	3.2 ± 0.1
B	—	Heparin	33	12 ± 2	19 ± 3
B	—	Yeast RNA	300	8.8 ± 0.9	16 ± 0
B	—	Poly(U)	300	15 ± 1	24 ± 5
B	Spermidine added	—	—	1.2 ± 0.1	2.7 ± 0.3
B	Spermidine added	Poly(U)	300	2.4 ± 0.4	5.3 ± 0.6
B	Spermidine, Ca^{2+} and Mn^{2+} added	—	—	0.8 ± 0.2	2.2 ± 0
B	Spermidine, Ca^{2+} and Mn^{2+} added	Poly(U)	300	1.5 ± 0.1	2.0 ± 0.4

Fig. 2. *Effect of heparin on size distribution of supernatant RNA*

Sucrose-density gradients of nuclear RNA (Δ) and of the RNA released into media A ($\bullet$) and B ($\blacktriangle$) containing ribonuclease inhibitor, after a 30min incubation in the presence of 2.0mM-ATP and 33 μg of heparin/ml. Results are means of two experiments each done in triplicate. The same standards were used as in Fig. 1.

Discussion

The results shown in Fig. 1(b) suggest that nuclear RNA is protected against degradation *in vitro* by serine-proteinase inhibitors. This is consistent with the results from several laboratories, which show that fibrous ribonucleoprotein skeletal structures (nuclear matrices) can be isolated from a variety of cell types, with the nuclear RNA more or less intact,

only if serine-proteinase inhibitors are present (Miller *et al.*, 1978; Herman *et al.*, 1978; Long *et al.*, 1979; Agutter & Birchall, 1979; Kaufmann *et al.*, 1981; Van Eekelen & Van Venrooij, 1981). Inhibition of RNA degradation by these means does not markedly affect either ATP-dependent RNA efflux or the slight ATP-independent efflux of low-molecular-weight material (Fig. 1a). However, this evidence does not eliminate the possibility that specific, ATP-stimulated, proteinases which are not inhibited by phenylmethanesulphonyl chloride and *N*-ethylmaleimide play a part in the efflux process. If such enzymes exist, their activity does not seem to lead to significant degradation of the nuclear RNA, and it is possible that they play a part in RNA transport *in vivo*. The inhibition of the NTPase and of ATP-dependent RNA efflux by phenylmethylsulphonyl fluoride, though not by the chloride, is surprising, particularly because Palayoor *et al.* (1981) found that the former did not affect RNA efflux. However, these workers used a medium containing a high concentration of homologous cytoplasmic protein, and the phenylmethanesulphonyl fluoride may well have reacted with components of this mixture rather than with the RNA-transport apparatus in the nuclei. It is possible that phenylmethanesulphonyl fluoride generates F^- in high local concentrations around the dephosphorylation site of the NTPase: it is known that F^- , by inhibiting dephosphorylation (Steer *et al.*, 1979; McDonald & Agutter, 1980), inhibits the NTPase (Clawson *et al.*, 1978). In view of the results, the design of future RNA-efflux experiments

might be improved by the inclusion of phenylmethanesulphonyl chloride in the medium.

ATP-stimulated efflux is not prevented by pre-treatment of the nuclei with a non-ionic detergent (Table 2), and this is consistent with the finding that the nuclear-membrane lipid plays no part in the efflux process in mammalian nuclei (Stuart *et al.*, 1977; Clawson & Smuckler, 1978; Agutter & Suckling, 1982a). However, the slight increase in ATP-independent efflux (Table 2) may indicate some damage to the apparatus responsible for RNA restriction to the nucleus, and in view of the evidence presented in the present paper and by Jacobs & Birnie (1982) that RNA adsorption to the nuclear surface does not markedly affect the results, it seems preferable to work with intact rather than membrane-denuded nuclei in experiments of this kind.

The 'stimulating' effect of exogenous RNA on efflux (Sato *et al.*, 1977; Ishikawa *et al.*, 1978) has been variously interpreted. The results presented in Table 3 indicate that stimulation of the NTPase does not account for the effect. Poly(U), unlike poly(A), poly(G) and total yeast RNA, does not affect this enzyme significantly (Agutter *et al.*, 1977), but it causes nuclear lysis in media which do not contain nuclear stabilizers (Goidl *et al.*, 1975). Heparin, which has an effect on efflux similar to that of poly(U), is among the acidic polymers that disrupt nuclear contents and cause both nuclear swelling and random leakage of nuclear material (Coffey *et al.*, 1974). The effect of exogenous RNA is probably attributable to this random disruptive effect on nuclei. The prevention of it by spermidine, Ca^{2+} and Mn^{2+} supports the view (Yu *et al.*, 1972; Agutter, 1980) that these cations should be present in any medium used for RNA-efflux experiments. If neither exogenous RNA nor cytoplasmic ribonuclease inhibitor is included in the medium, there is marked loss of trichloroacetic acid-precipitable label, particularly in the supernatant, presumably because of ribonuclease action (Table 3).

Because the results described in the present paper seem to answer the three methodological criticisms listed in the introduction, and because other methodological criticisms have been answered by previous studies (e.g. Schumm & Webb, 1974; Sato *et al.*, 1977; Agutter, 1980, 1982), it may reasonably be concluded that 'medium A' (Agutter & McCaldin, 1979) is adequate for studies of RNA efflux from rat liver nuclei that are intended to elucidate the general mechanism of nucleo-cytoplasmic RNA transport. It cannot be concluded that the same medium will be adequate for the study of RNA efflux from nuclei from other sources. Moreover, although it is clear that the labelled RNA that appears in the supernatants of the incubations described here is not a random fraction of degraded

or undegraded nuclear RNA, but predominantly resembles rat liver mRNA (see Agutter, 1982), it is not clear that it is entirely mRNA: indeed, it need not necessarily be so for mechanistic studies of the kind undertaken in my laboratory to be valid. However, if the efflux of specific mRNA species is to be studied, more stringent methodological criteria are involved, and to establish these, techniques such as those used by Jacobs & Birnie (1982) are necessary. It is therefore noteworthy, in the light of the results described in the present paper, that Jacobs & Birnie (1982) found the medium of Yu *et al.* (1972) [essentially medium A supplemented with cytosol proteins and an ATP-regenerating system (cf. Agutter, 1982)] to be adequate, at least for studying the processing and efflux of mRNA species in high- and intermediate-abundance classes. Details of the processing of specific gene products could also be studied productively by using RNA-efflux methods. The most suitable nuclei for such studies would presumably be obtained from a type of cell in which (a) the total cytoplasmic mRNA is dominated by a few well-characterized species, (b) the nuclear precursors of at least some of these high-abundance messengers are also well characterized, (c) the distribution and relative abundances of these major transcription products change markedly and predictably with conditions (e.g. with stages in embryonic development). However, the adequacy of the incubation medium used for such nuclei would first require to be established according to the criteria adopted by Jacobs & Birnie (1982), preferably by using similar techniques, and not according to the criteria and by the techniques described in the present paper.

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