The Use of Spermidine for the Isolation of Nuclei from Mouse Liver. Studies of Purity and Yield During Different Physiological Conditions

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A 15 min method for the isolation of clean liver cell nuclei from normal, starved, hepatectomized and sham operated mice in mediums not containing bivalent cations is described. The isolation medium contained 0.5 mM spermidine for stabilization of the nuclear membrane, and macaloid and EDTA for inhibition of RNase activity. The RNA/DNA ratio in normal liver cell nuclei was 0.25–0.29 and the protein/DNA ratio 5.2. The RNA/DNA ratio was 0.23–0.25 after starvation for 24 hours and increased to 0.5 at 72 and 144 hours after partial hepatectomy.

Introduction

In order to isolate cell nuclei from mammalian tissues there are essentially two ways of achieving the separation of nuclei from cytoplasm. One method involves the use of detergents often in a citric acid buffered medium and short time, low speed centrifugation. Another, more time consuming procedure, is to liberate the nuclei from cytoplasmic contamination by suspension in a hypertonic sucrose solution and high speed gradient centrifugation [1–3]. The amount of protein and nucleic acid recovered in the isolated nuclei and the denaturation of their chromatin are very much dependent on the homogenisation medium used and the centrifugation method adopted [4]. Moreover, a method suitable for normal tissues may be less satisfactory for tissues in a different physiological stage, for example tumour tissues [3, 5]. Ca^{2+} and Mg^{2+} [3, 5] are widely used as stabilizors of the nuclear membrane during isolation experiments. On homogenising in media containing bivalent cations we found however a much firmer adherance between nuclei and cytoplasm in regenerating liver than in normal liver [7]. Furthermore, both Ca^{2+} and Mg^{2+} [8] have been found to accumulate in nuclei when present in the homogenate during the isolation procedure. These ions cause changes in the activities of nucleases [9] and in the ability of the nuclei to synthesize different kinds of RNA [10] *in vitro*. Therefore we have tried to find a method for the isolation of cell nuclei from mouse liver in different physiological conditions without the addition of bivalent cations. The method should be simple and rapid and give a high and reproducible yield of nuclei suited for the analysis of undegraded RNA and DNA. We have chosen an EDTA-critric acid buffered medium, pH 3.3, to which spermidine was added for the stabilization of RNA and the nuclear membrane [11, 12] and macaloid for the inhibition of RNase activity [13]. The detergent Trition N 101 was added to the medium, enabling the isolation of pure nuclei by low speed centrifugation for 15 min. Isolated nuclei from livers of normal, starved, sham operated and hepatectomized animals were analyzed for their content of DNA, RNA and protein. The percentage amount of liver DNA recovered in the nuclear pellet was calculated in each experiment. The effects of using a higher pH and of introducing gradient centrifugation on the results of the analysis of the isolated nuclei were investigated.

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Material and Methods

Animals

Young male mice, weighing 25–30 g were used for the experiments. Housing, feeding and operation of the animals have been described previously [4]. All operations were made in series. Each series comprised 1–6 normal animals and groups of 1–6 animals killed at 12, 24, 48, 72 and 144 hours after partial hepatectomy or sham operation. The livers were dissected out and frozen in liquid N_2. They were used for preparation immediately or kept frozen 2–4 days at –25 °C.

Isolation of nuclei

One gram of liver from one mouse or from the pooled livers of 2–3 mice was homogenized in

8 ml of medium A (0.025 M citric acid, 25 mM KCl, 1 mM EDTA, 0.5 mM spermidine (Calbiochem), 50 mg Macaloid L (Baroid Division, Texas), 0.1 M sucrose adjusted to pH 3.3 with 0.2 M Tris) in a Potter Elvehjem homogenizer with a motordriven teflon pestle. Ten to twelve strokes at 900 rpm were needed for complete homogenisation. The homogenate was filtered through a nylon sieve in a millipore filter equipment connected to a syringe. Then 16 ml of solution B (as solution A but 0.32 M sucrose) were added to the homogenate by means of the syringe and the nylon sieve. The nuclei were sedimented by centrifugation for 5 min at $900 \times g$. The pellet was washed thoroughly with 25 ml of solution B, to which was added 0.3% Triton N 101. The medium was added by way of the syringe and nylon sieve, which were thus also washed. The nuclei were sedimented again by centrifugation for 5 min at $900 \times g$. All steps were carried out at 0 – 4 °C.

To test the possible absorption of cytoplasmic RNA in the nuclei at different spermidine concentrations the supernatant containing cytoplasm obtained from the first centrifugation of a homogenate of labelled liver tissue was used for the isolation of nuclei from unlabelled liver. Labelling of the liver tissue was achieved by giving the mice [^{3}H]uridine two hours prior to the experiments.

In three experiments with liver from normal mice the nuclear pellet from the first centrifugation was suspended in solution B containing 2 M sucrose and sedimented through clean 2 M sucrose in solution B by centrifugation at $40\,000 \times g$ for an hour. Thus the washing procedure with Triton N 101 was excluded.

In three experiments with liver from normal mice the pH of the isolation medium was buffered to pH 7.2 using Tris-HCl. Thus solution A was replaced by solution C (0.05 M Tris HCl, pH 7.2, 10 mM KCl, 1 mM EDTA, 0.5 mM spermidine, 2.8 mM KH_2PO_4, 50 mg macaloid/l and 0.1 M sucrose). Solution B was replaced by solution D (as solution C but 0.32 M sucrose). After the first sedimentation the nuclei were washed in a medium containing 0.3% Triton N 101, 0.25 M sucrose, 0.05 M Tris-HCl pH 7.2, 0.5 mM spermidine, 2.8 mM KH_2PO_4.

Analysis of yield, nuclear protein, RNA and DNA

The yield was defined as the amount of DNA recovered in the isolated nuclei calculated as a percentage of the total DNA in the homogenate (sum of DNA on the filter sieve, in the supernatants and in the nuclear pellet). The total amount of DNA per gram of normal liver was also determined by direct analysis of liver tissue from 12 normal mice. Nucleic acids were determined essentially according to Munro and Fleck [15]. The total hydrolysis of RNA after 1 hour at 37 °C was checked by another hydrolysis for 15 hours at 37 °C. RNA was determined by UV absorption using highly polymerized RNA (BDH) as a standard. DNA was determined according to Burton [15, 16] using thymus DNA (Sigma) as a standard. The amount of nuclear proteins was measured according to Lowry *et al.* [17] with bovine serum albumin as a standard. For analysis with light microscopy the nuclei were stained with Türk's reagent and for electron microscopy they were prepared according to Maggio *et al.* [18].

Results

The sum of the amounts of DNA found in the different fractions per gram of normal liver was equivalent to the amount of DNA per gram liver found by direct analysis, *i. e.* 1.88 mg/g liver. With the standard procedure used, 79 – 88% of the DNA in liver homogenates from normal mice was recovered in the nuclear pellet (Table I). 10 – 17% of the DNA remained in the supernatant and 2 – 5% on the filter. The distribution of the DNA in the different fractions was not altered by starvation for 24 hours, or by partial hepatectomy or sham operation.

The suspending of the nuclear pellet in 2 M sucrose added by way of the filter after the first centrifugation increased the percentage amount of DNA recovered on the filter (Table I). The gradient centrifugation increased the amount of DNA recovered in the supernatants. When the pH of the isolation medium was increased to 7.2 an increased percentage amount of DNA was also recovered in the supernatants (Table I).

The RNA/DNA ratio was 0.25 – 0.29 in the nuclear pellet isolated from normal mice by the 15 min long standard procedure (Table I). This ratio was decreased by starvation. It was increased 48, 72 and 144 hours after partial hepatectomy and also after a sham operation, though to a lesser extent. The RNA/DNA ratio in the nuclear pellet recovered from livers of normal mice by gradient centrifugation was 0.18 – 0.20. The time required for the isolation of nuclei using this method was 75 min. An increase in the pH of the isolation medium to 7.2 did not appreciably change the RNA/DNA ratio (Table I).

When nuclei were isolated using supernatants from homogenates of labelled livers, radioactivity

Table I. Amount of DNA in the different fractions from one gram of liver in normal, starved, sham operated and hepatectomiced mice. Unfractionated liver from normal mice contained 1 880 mg DNA per gram wet weight. Amount of RNA in the nuclear pellets. Mean ± S.E.

Mouse No.	Treatment of animal	DNA						RNA	
		In nuclear pellet		In supernatants		On filter		In nuclear pellet	RNA/DNA
		[μg]	[per cent of total]	[μg]	[per cent of total]	[μg]	[per cent of total]	[μg]	
		0.5 mM spermidine, pH 3.3, 15 min preparation							
1	Normal	1501	85	205	12	58	3	394	0.26
2	Normal	1650	88	193	10	32	2	478	0.29
3	Normal	1376	79	296	17	69	4	382	0.28
4	Normal	1463	81	275	15	74	4	381	0.26
5	Normal	1552	81	259	14	92	5	384	0.25
6	Normal	1509	83	263	14	54	3	389	0.26
	Mean ± S.E.	1508±37	83±3	249±17	14±1	63±8	3±0.5	401±15	0.27±0.01
7	Starvation 24 h	1542	85	220	12	47	3	360	0.23
8	Starvation 24 h	1563	86	187	10	72	4	376	0.24
9, 10, 11 pooled	6 h	1379	76	355	20	79	4	402	0.29
12, 13, 14 pooled	12 h after	1450	83	232	13	61	4	395	0.27
15, 16, 17 pooled	24 h partial	1393	79	312	18	55	3	416	0.30
48, 19, 20 pooled	48 h hepatec-	1543	82	292	16	46	2	549	0.36
21, 22 pooled	72 h tomy	1668	82	330	16	50	2	1045	0.63
23, 24 pooled	144 h	1810	84	292	13	65	3	1016	0.56
25	6 h	1500	79	310	16	90	5	443	0.30
26	12 h after	1589	81	305	16	58	3	442	0.28
27	24 h sham	1525	82	285	15	45	3	485	0.32
28	48 h opera-	1590	85	202	11	67	4	479	0.30
29	72 h tion	1555	86	206	11	50	3	477	0.31
30	144 h	1508	84	216	12	74	4	448	0.30
		0.5 mM spermidine, pH 3.3, 75 min preparation, sucrose gradient							
31	Normal	1229	70	408	23	127	7	242	0.20
32	Normal	1286	71	394	22	124	7	239	0.19
33	Normal	1161	64	508	28	147	8	207	0.18
		0.5 mM spermidine, pH 7.2, 15 min preparation							
34	Normal	1440	78	337	18	72	4	455	0.32
35	Normal	1125	62	582	32	95	5	275	0.24
36	Normal	1260	71	436	24	87	5	351	0.28

was absorbed in the nuclear pellets if the spermidine concentration of the medium was 0.8 mM but not appreciably if it was 0.5 mM or less.

The contents of DNA RNA and protein in the nuclear pellets from livers of partially hepatectomized mice were analyzed in 5 operative series.

The amounts of DNA and protein recovered in the nuclear pellets (per g of wet weight) from livers of partially hepatectomized mice 6 – 24 hours after the operation were around 90% of the values found in normal mice (Fig. 1). At 48 hours post operati-

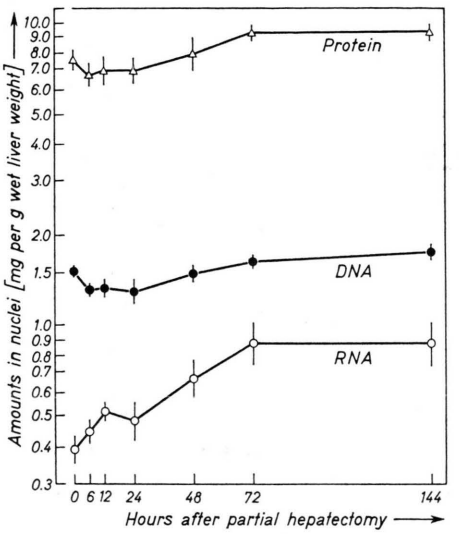

Fig. 1. Amounts of DNA, RNA and protein in nuclear pellets from one gram of liver (wet weight) after partial hepatectomy in mice. Mean ± S.E. Each point represents the mean from at least 10 mice operated in 5 different series. Semilogarithmic scale.

vely the contents of protein and DNA in the nuclear pellets had increased to the normal level. At 72 and 144 hours after partial hepatectomy the amounts were about 115% of the normal values.

The amount of RNA recovered in the nuclear pellet increased after partial hepatectomy, Fig. 1. At 72 and 144 hours after the operation the nuclear pellets contained twice the normal amount of RNA.

Light microscopic examination of the nuclear pellets indicated that the isolated nuclei were intact and little contaminated by whole cells and cytoplasmic structures. A representative sample of normal liver nuclei obtained using the standard procedure and stained with Türk's reagent is shown in Fig. 2 *. As viewed under the electron microscope, nuclei isolated at pH 3.3 had condensed chromatin (Fig. 3 A), whereas greatly reduced chromatin condensation was seen at pH 7.2 (Fig. 3 B).

* Figs 2 and 3 see Tables on pages 270 a and b.

Discussion

The procedure used for the isolation of nuclei in this report gave clean nuclei at a high and reproducible yield from normal as well as from regenerating livers in the short time of 15 min. It can be carried out at either pH 3.3 or pH 7.2. Furthermore, it has the advantage of excluding bivalent cations from the preparation, thereby reducing nuclease activity and rendering possible *in vitro* analysis of different kinds of RNA synthesis. Changes known to occur in the amount of nuclear RNA after starvation [19, 20] or partial hepatectomy [20, 22] were preserved during the isolation procedure.

Of the total amount of DNA in a normal liver homogenate 79 – 88% was recovered in the nuclear pellet and should originate from the parenchymal and endothelial tissues of the liver. At filtration of the homogenate 3 – 5% of the DNA was caught on the filter and probably originated from liver connective tissue. Unsedimented nuclei, nuclear fragments and possibly mitochondria undoubtedly contributed to the 10 – 17% of the liver DNA recovered in the supernatants.

When the isolation medium contained 2.0 M sucrose, the filter was obviously not so well rinsed from nuclear material during the preparation. More nuclear material also remained in the supernatant at centrifugation.

The percentage yield of DNA in the nuclear pellet using our method was in the same range as that found for normal liver by Blobel and Potter [6]. When using the Blobel and Potter method in a previous study [24] we found a decreased sedimentability of DNA in regenerating liver as compared to normal liver. It was found to depend on the presence of many unbroken cells and very large nuclei, which did not penetrate the sucrose gradient. The EDTA isolation media not containing Ca^{2+} and Mg^{2+} used in the present study greatly reduced the frequency of unbroken cells in homogenates from regenerating liver. Also, omission of the gradient step facilitated the sedimentation of large nuclear particles.

A low protein/DNA or RNA/DNA ratio is often considered to be a criterion for clean nuclear pre-

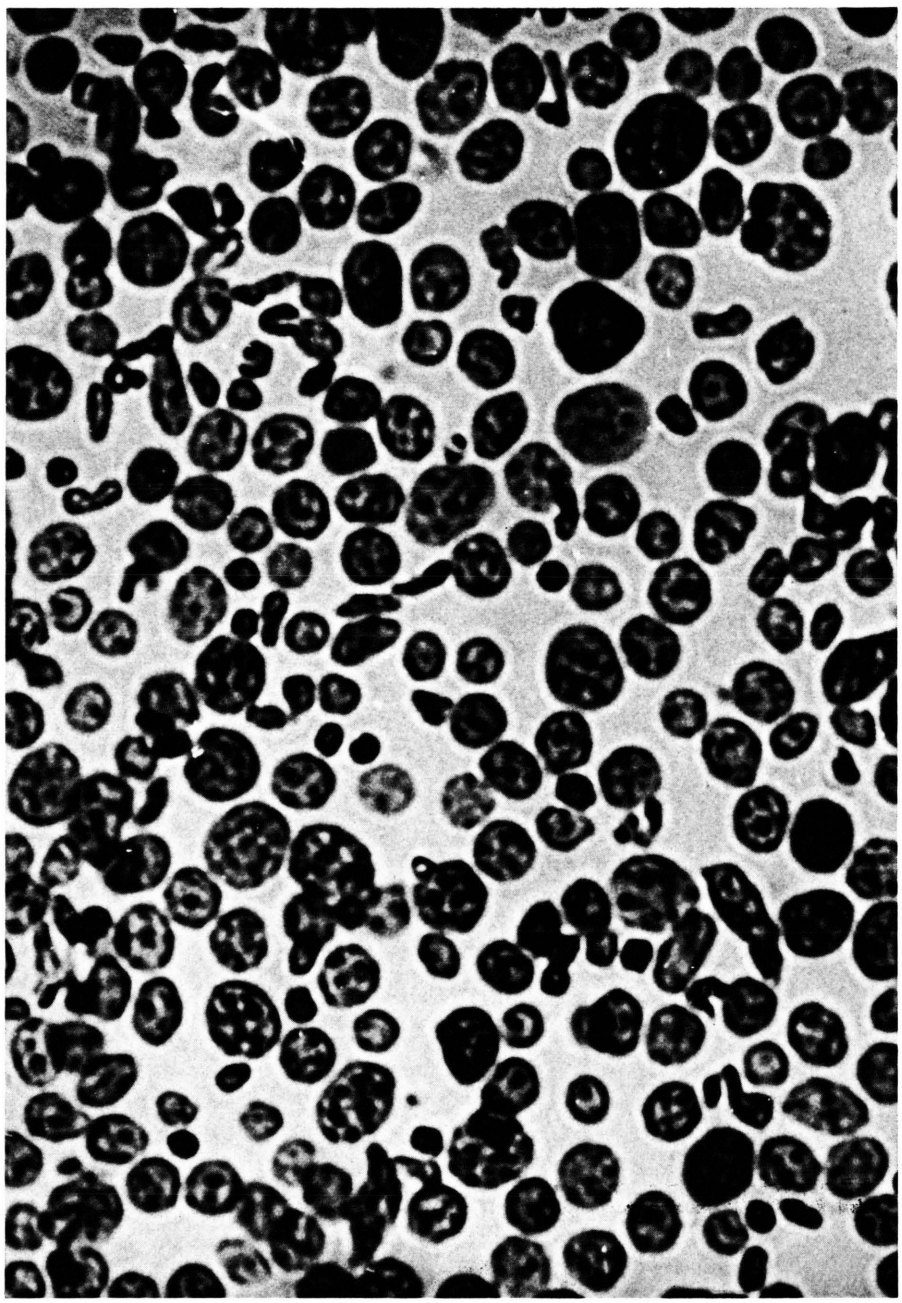

Fig. 2. Light microscopic photograph of liver nuclei isolated at pH 3.3 from normal mice. Magnification 600.

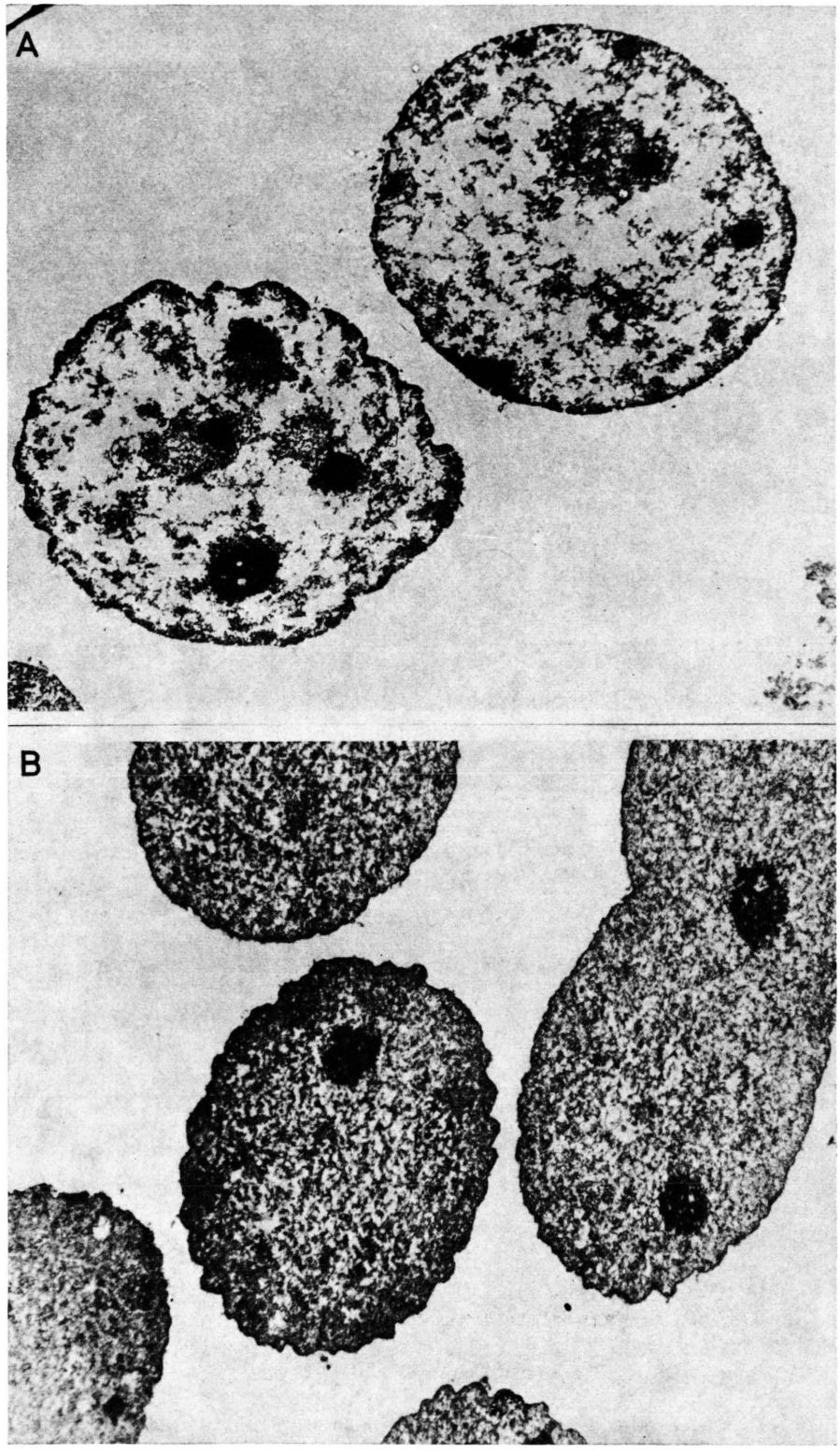

Fig. 3. Electron micrograph of isolated nuclei from normal liver. Magnification 8 800. A. pH of the isolation medium 3.3, B. pH of the isolation medium 7.2.

paration. The considerable influence of proteolytic activity on the protein/DNA ratio is well known [25]. When the main procedure for the isolation of nuclei was used in this study the protein/DNA ratio in the nuclear pellet from normal liver was 5.2. This value is in the same range as that found with other aqueous procedures [2, 25].

The RNA/DNA ratio in the nuclear pellet from normal liver, 0.25 – 0.29, could be decreased to 0.18 – 0.20 by sedimentation of the nuclei through 2.0 M sucrose. The ultracentrifugation may liberate the nuclei from cytoplasmic contamination more effective than low speed centrifugation. However, it cannot be excluded that endogenous nuclease activity or leakage or migration of nuclear RNA to the isolation medium during the 75 min long procedure contributes to the low RNA/DNA ratio.

The RNA/DNA ratios in isolated liver nuclei reported in other studies vary from 0.11 – 0.13 [26, 6] to 0.21 – 0.27 [22, 2, 3]. They should be greatly influenced not only by the isolation method but also by the methods and standards used for the analysis of the nucleic acids. The RNA/DNA ratio in the nuclear pellets from normal, starved and hepatectomized mice in this study is higher than that found by other investigators. However, the clean appearance of the cell nuclei and the fact that absorption of cytoplasmic RNA could not be found led us to believe that the inhibition of nuclease activity as well as the good preservation of the nuclei have contributed to the rather high RNA/DNA values found in this study. Thus, we hope that the isolated nuclei are well suited for analyses of undegraded nuclear RNA.

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