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Accumulation of the isolated carboxy-terminal domain of histone H1 in the *Xenopus* oocyte nucleus

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Histone H1 accumulates in the nucleus after injection into the cytoplasm of *Xenopus* oocytes. A proteolytic fragment of 89 amino acids encompassing the carboxy-terminal domain also accumulates in the nucleus. Lysine, alanine and proline compose 84% of this domain. Accumulation is not due solely to the high lysine content since poly-L-lysine does not accumulate in the nucleus when injected into the cytoplasm of *Xenopus* oocytes. Proteolytic fragments encompassing other domains of the molecule are degraded in the oocyte after injection. In these instances degradation is more rapid in the cytoplasm than in the nucleus giving the false impression of accumulation in the nucleus, an artefact which is likely to confuse other studies of protein migration. Susceptibility to rapid degradation is a dominant feature, thus the globular domain destabilises the contiguous carboxy-terminal domain. The properties of the carboxy-terminal domain of H1 and the possible involvement of the amino acids lysine, proline and alanine in migration are dicussed and compared with those of a domain that specifies migration of nucleoplasmin into the oocyte nucleus.

Key words: oocyte/H1/subfragment/nuclear accumulation

Introduction

Gurdon (1970) demonstrated that the mechanism of accumulation of nuclear proteins within the cell nucleus can be investigated by injecting radiolabelled histones into the cytoplasm of *Xenopus* oocytes. Subsequently Bonner (1975a, 1975b) demonstrated intranuclear accumulation for the individual histones H4, H2B, H2A and H1 and for other nuclear proteins extracted from radiolabelled oocytes. In contrast, proteins extracted from the cytoplasm remained cytoplasmic after injection.

These experiments demonstrated that uptake of proteins into the nucleus is selective and that the mature protein rather than some precursor form possesses all the features necessary for entry and accumulation. The mechanism of uptake and the basis of the selectivity are unknown. We have recently shown that in the case of nucleoplasmin (Earnshaw *et al.*, 1980) the ability to accumulate in the nucleus is conferred on the molecule by a particular domain which is acidic and hydrophilic (Dingwall *et al.*, 1982). In this instance accumulation appears to arise from selective entry through the nuclear envelope rather than from free diffusion into the nucleus followed by selective binding. We have subjected histone H1 from calf thymus to a similar experimental analysis by assaying isolated proteolytic fragments of H1 for their ability to accumulate in the nucleus of *Xenopus* oocytes to ask if a similar discrete domain is involved in the accumulation of other nuclear proteins and, if so, how does its structure compare with the equivalent domain of nucleoplasmin.

Results

Accumulation of histone H1 in the oocyte nucleus

Figure 1 is an autoradiograph of an SDS-polyacrylamide gel and shows that calf thymus histone H1 accumulates within the nucleus after microinjection into the cytoplasm of mature oocytes of *X. laevis*. The nuclear to cytoplasmic concentration ratios were calculated assuming that the nucleus represents 12% of the aqueous volume of the oocyte (Bonner, 1978) and shows the extent of accumulation at the various times after injection. This result with a single pure histone confirms the results of Gurdon (1970) and Bonner (1975a) which we outlined above. In order to determine whether this property is conferred on H1 by a particular domain we employed a strategy similar to that used for nucleoplasmin (Dingwall *et al.*, 1982), namely that of microinjecting radiolabelled proteolytic fragments of the protein into the cytoplasm of fully grown oocytes and observing whether they can accumulate within the nucleus.

The structural domains of histone H1

Histone H1 has three distinct structural domains which have been demonstrated experimentally and which parallel those suggested on the basis of the distribution of amino acids (Cole, 1977). These domains are as follows. The amino-

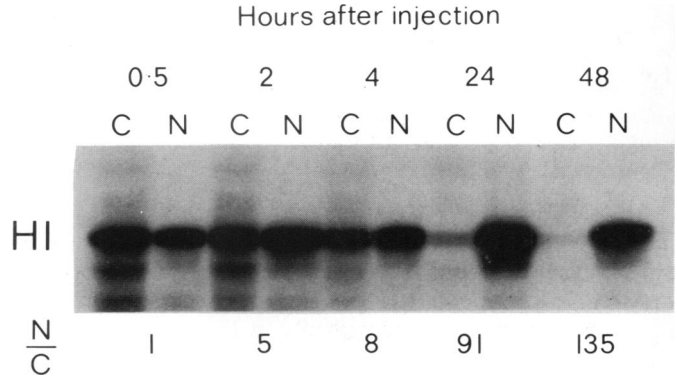

Fig. 1. Accumulation of histone H1 in the oocyte nucleus. Radiolabelled calf thymus histone H1 was microinjected into the cytoplasm of fully grown oocytes of *X. laevis*. Each oocyte was injected with 2 ng of histone at a specific activity of 270 c.p.m./ng in a volume of 50 nl. The oocytes were then incubated in modified Barths saline at 20°C and the nuclei were isolated manually at the indicated times. The separated nuclei and cytoplasms were analysed by SDS-polyacrylamide gel electrophoresis and the gels were autoradiographed. N/C = nuclear to cytoplasmic concentration ratio assuming that the cytoplasmic volume is 8.3x the nuclear volume (Bonner, 1978). A 12 h time point gave a nuclear to cytoplasmic concentration ratio of 47.

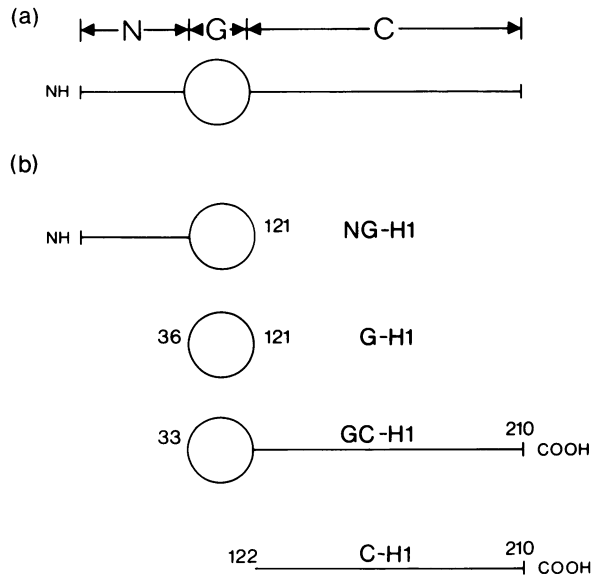

Fig. 2. Schematic representation of the structural domains of histone H1 and the proteolytic fragments. **(a)** Structural domains. N, amino-terminal domain; G, globular domain; C, carboxy-terminal domain. **(b)** Proteolytic fragments of histone H1. NG-H1, amino-terminal-plus-globular domain fragment comprising residues 1 – 121; G-H1, globular domain fragment comprising residues 36 – 121; GC-H1, globular-plus-carboxy-terminal domain fragment comprising residues 33 – 210; C-H1, carboxy-terminal domain fragment comprising residues 122-210.

terminal domain stretches from the amino terminus for at least 34 residues and is ~21% lysine. Analysis of H1 subtypes has revealed great variability in the length and sequence of this region. This part of the molecule is thought to be largely unstructured in solution (Bohm *et al.*, 1982). The central globular domain involves approximately the next 80 residues. It is thought to be folded, showing ~28% alpha helix and is the most hydrophobic domain of H1 with a high degree of sequence conservation. The remainder of the molecule comprises the carboxy-terminal domain and represents almost half the molecule. One third of the residues in this domain are lysine so this region contains ~68% of all the lysine residues in the protein. The amino acids lysine + proline + alanine make up 84% of the total amino acid composition of this region. Like the amino-terminal domain it is thought to be in a highly extended, unstructured conformation in solution (Bohm *et al.*, 1982).

The proteolytic fragments of histone H1

The proteolytic fragments used for injection were prepared using published procedures (Chapman *et al.* 1976; Hartman *et al.*, 1977; Bohm *et al.*, 1982). The structural domains they encompass are shown schematically in Figure 2. Thrombin cleaves histone H1 at a single site (Chapman *et al.*, 1976) and was used to produce two fragments, NG-H1 (residues 1 – 121) encompassing the amino-terminal and globular domains, and C-H1 (residues 122 to the carboxy terminus at residue 210) the carboxy-terminal domain fragment.

G-H1, the globular domain fragment (residues 36 – 121) was purified from a trypsin digest of intact H1 (Hartman *et al.*, 1977). Submaxillary protease was used to produce GC-H1, the fragment encompassing the globular and carboxy-terminal domains (residues 33 – 210) (Bohm *et al.*, 1982). The purified fragments were homogeneous as judged by SDS-polyacrylamide gel electrophoresis.

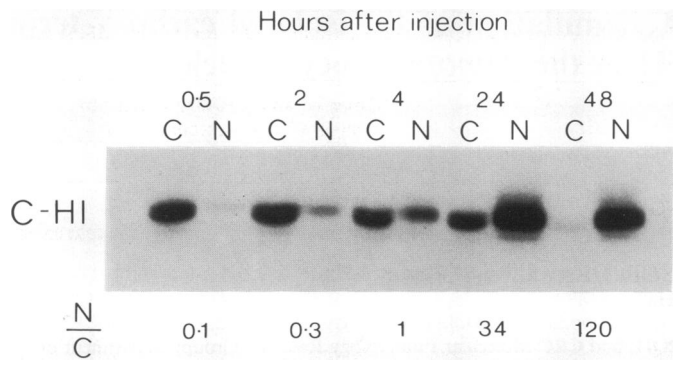

Fig. 3. Accumulation of the carboxy-terminal domain fragment (C-H1) in the oocyte nucleus. The radiolabelled carboxy-terminal domain fragment (C-H1) of calf thymus histone H1 was microinjected into the cytoplasm of fully grown oocytes of *X. laevis*. Each oocyte was injected with ~2 ng of protein labelled to a specific activity of 250 c.p.m./ng in a volume of 50 nl. The ooyctes were incubated in Barths saline at 20°C and the nuclei were isolated manually at the indicated times. The separated nuclei and cytoplasms were analysed by SDS-polyacrylamide gel electrophoresis and the gels were autoradiographed. N/C = nuclear to cytoplasmic concentration ratio assuming that the cytoplasmic volume is 8.3x the nuclear volume.

Assay of purified fragments for accumulation in the nucleus

The pure fragments described above were labelled and microinjected individually into oocytes and analysed exactly as described for intact H1 (see Materials and methods).

The carboxy-terminal domain fragment C-H1 accumulates in the nucleus. This fragment (C-H1) is stable in the oocyte (no detectable degradation occurs in 48 h) and accumulates in the nucleus (Figure 3). A comparison of the nuclear to cytoplasmic concentration ratios for this fragment and intact H1 at various times after injection (compare Figures 1 and 3) indicates that this fragment accumulates in the nucleus less than intact H1 does in the first 24 h, but at 48 h both have accumulated to approximately the same extent. This result implies that this domain plays a role in the accumulation of histone H1 in the nucleus. However, some other feature of the molecule may also be involved in the accumulation of H1 since migration of the fragment appears slower than that of intact H1.

The amino-terminal globular domain fragment (NG-H1) and the globular carboxy-terminal domain fragment (GC-H1) are unstable. These two fragments were found to behave in a similar manner to one another. At the first time point (30 min after injection) a proportion (16 – 21%) of the injected fragments had entered the nucleus and was stable there; approximately one third of the material present in the nucleus at the first time point was still present 24 h later, i.e., 5 – 7% of the material injected initially. The bulk of the injected fragment was degraded rapidly in the cytoplasm.

The rate of disappearance of each fragment from the oocyte was determined from densitometer traces of the autoradiographs and it follows first order kinetics. The half life was determined (see Table I) and used to calculate the nuclear to cytoplasmic concentration ratios at the early time points assuming that the concentration ratio is 1 at zero time (i.e., instantaneous equilibration of the injected material between the nucleus and cytoplasm). The relatively slow loss of material from the nucleus was not taken into account in these calculations as they were performed only for the first 4 h after injection. The concentration ratios calculated in this way were compared with the concentration ratios observed (see

Table I. Nuclear to cytoplasmic concentration ratios for NG-H1, G-H1 and GC-H1 fragments of histone H1

	Observed and predicted nuclear to cytoplasmic concentration ratios		
	0.5 h	2 h	4 h
NG-H1	2.6	5.1	8.5 observed
(Half life 1.25 h)	1.3	3	9 calculated
G-H1	0.5	1.5	2.7 observed
(Half life 0.7 h)	1.6	7.2	52 calculated
GC-H1	1.6	2.5	3.1 observed
(Half life 1.8 h)	1.2	2.2	4.7 calculated

The accumulation of the proteolytic fragment shown was examined using the protocol described for histone H1 and the fragment C-H1. The concentration ratios determined from densitometer traces of the autoradiographs (observed – three separate experiments) are compared with those calculated assuming equilibrium between nucleus and cytoplasm to give a nuclear to cytoplasm concentration ratio of 1 and degradation in the cytoplasm (first order kinetics) at the observed rates. NG-H1, amino-terminal plus globular domain fragment; G-H1, globular domain fragment; GC-H1, globular plus carboxy-terminal domain fragment.

Table I).

For the two fragments NG-H1 and GC-H1, the observed concentration ratios are not significantly different from those calculated. That is, the observed data can be explained by proposing that the injected fragment equilibrates rapidly between the nucleus and cytoplasm afer injection but is degraded in the cytoplasm.

The greater instability of these fragments in the cytoplasm compared with the nucleus has an important consequence. Substantial, apparent accumulation in the nucleus is observed 4 h after injection (see Table I). Therefore, if observations had been made only at longer periods after injection, the fragments would have apparently accumulated in the nucleus to a significant extent. Only by monitoring the total amount of radioactivity in the oocyte from the earliest time point can this potential artefact be avoided. This artefact could seriously confuse other studies of intranuclear migration, notably those which use fluorescent antibodies to monitor nuclear:cytoplasmic ratios in cultured cells. When this artefact is allowed for, the only fragment which is seen to accumulate in the nucleus is C-H1.

The globular domain fragment G-H1. This is the least stable fragment and it does not even reach the concentration ratios expected for equilibration followed by degradation. This may be explained by non-specific binding of the fragment in the cytoplasm such that it is unable to equilibrate between the nucleus and cytoplasm before it is degraded, or alternatively, it could be due to exclusion by the nuclear envelope. Alternatively it could be explained by degradation within the nucleus. Unlike the fragments NG-H1 and GC-H1, the small amount of material which entered the nucleus does not persist but disappears as rapidly as the material in the cytoplasm.

The globular domain destabilises the carboxy-terminal domain

The isolated carboxy-terminal domain fragment (C-H1) is stable in the oocyte but the globular plus carboxy-terminal domain fragment (GC-H1) is completely unstable such that the carboxy-terminal domain fragment was not observed as a product of the degradation of GC-H1 within the oocyte. This indicates that the attachment of an unstable protein domain (the globular domain) to a stable protein domain results in its destabilisation.

Table II. Nuclear to cytoplasmic concentration ratios for poly-L-lysine

	Nuclear to cytoplasmic concentration ratios		
Mol. wt. x 10^{-3}	4 h	24 h	48h
4	–	–	0.3
8.3	0.03	0.03	0.06
26	0.03	0.04	0.1
41	0.13	0.04	0.13

Poly-L-lysine of four mol. wts. were radiolabelled and microinjected into fully grown oocytes of *X. laevis*. Each oocyte was injected with 3–4 ng of each polymer at a specific activity of 300–400 c.p.m./ng. The oocytes were then incubated in Barths saline at 20°C and the nuclei were isolated manually. The isolated nuclei and cytoplasm were then counted in a Beckman Bio Gamma Counter and the concentration ratios calculated.

Inhibition of proteolytic activity in the oocyte

Attempts to inhibit proteolytic degradation in the oocyte and thereby stabilise the fragments were unsuccessful. Co-injection of the fragments with the inhibitors antipain, aprotinin, bovine pancreatic trypsin inhibitor, leupeptin or pepstatin or incubation in the presence of the anti-lysosomal agent chloroquine failed to stabilise the fragments.

Positive charge is not sufficient for accumulation in the nucleus

Histones are accumulated in the oocyte nucleus in large quantities for use in early development (Woodland and Adamson, 1977; Van Dongen *et al.*, 1983) probably in the form of complexes with acidic components of the nucleus (Laskey *et al.*, 1978; Kleinschmidt and Franke, 1982). Histone H1 is within the size range of molecules that have been shown to diffuse into the nucleus after injection into the cytoplasm (Paine and Feldherr, 1972), hence the accumulation of H1 could be the result of diffusion into the nucleus and retention there by binding to these acidic components. Histone H1 is highly basic comprising 27% lysine and the carboxy-terminal domain is 43% lysine. Histone H1 contains very little arginine (2%) none of which is in the carboxy-terminal domain. One would therefore expect that the lysine residues would provide the greatest contribution to the binding of histone H1 to acidic components of the nucleus.

The ability of poly-L-lysine to accumulate in the nucleus was therefore examined by microinjection into the oocyte of radiolabelled polymers of four different mol. wts. Two of the polymers are of very low average mol. wt. (4000 and 8300) and are therefore small enough to enter the nucleus by diffusion. The polymers are stable in the oocyte but none of them was able to accumulate in the nucleus (Table II). This was probably not due to aggregation in the cytoplasm as physicochemical studies of poly-L-lysine indicate that in solution at neutral pH and temperatures below 50°C the polymer assumes an alpha-helical conformation and does not form aggregates (Hartman *et al.*, 1974; Snell and Fasman, 1973). Inter-molecular association is favoured at high polymer concentrations but in these experiments 3–4 ng of each polymer was injected to give a concentration in the oocyte of ~3–4 μg/ml. Therefore it appears unlikely that the failure of poly-L-lysine to accumulate in the nucleus is due to aggregation. It may be due to binding to cytoplasmic com-

Table III. Amino acid compositions (mol%) of the carboxy-terminal domain fragment of histone H1 (C-H1) and the 12 000 mol. wt. tail fragment of nucleoplasmin

	C-H1	12 000 mol. wt. fragment
Asx	0.2	9.1
Thr	4.8	4.3
Ser	2.7	6.0
Glx	1.2	23.8
Pro	10.0	8.5
Gly	4.4	7.8
Ala	30.9	10.3
Val	2.5	3.4
Met	–	0.3
Ile	–	1.4
Leu	0.1	4.2
Tyr	–	0.4
Phe	–	1.2
His	–	1.0
Lys	43.2	15.6
Arg	–	2.6

ponents but this would presumably be a consequence of its charge and one may therefore expect the highly basic carboxy-terminal domain fragment to behave in a similar manner, but it does not.

Comparison of C-H1 and the 12 000 mol. wt. tail fragment of nucleoplasmin.

Both nucleoplasmin and H1 yield defined fragments which have the ability to accumulate in the oocyte nucleus after injection into the cytoplasm. Both fragments contain a large proportion of charged amino acids (see Table III), and both are extremely hydrophilic.

However their overall amino acid compositions are markedly different; the fragment C-H1 is extremely basic while the 12 000 mol. wt. tail fragment of nucleoplasmin is acidic (Dingwall *et al.*, 1982) and contains a much wider range of amino acids than the carboxy-terminal domain fragment of H1, though a small region of sequence homology is not excluded.

Discussion

We have shown here that intact histone H1 from calf thymus accumulates in the oocyte nucleus after microinjection into the cytoplasm. This confirms the results of Bonner (1975a). The *Xenopus* oocyte nucleus contains 30–40 ng endogenous histone H1 (Van Dongen *et al.*, 1983) and so our results show that the injected histone is accumulated against a substantial concentration gradient.

Histone H1 and hence any proteolytic fragments derived from it are small enough to enter the nucleus by free diffusion (Paine and Feldherr, 1972; Paine, 1975). Therefore we have looked for fragments which have the ability to accumulate in the nucleus to an extent greater than can be explained by equilibration between the nucleus and cytoplasm. We have found that a fragment which encompasses the carboxy-terminal domain has the ability to accumulate significantly in the nucleus. This fragment is extremely basic and has a very simple amino acid composition, yet its ability to accumulate in the nucleus cannot be explained by its positive charge as poly-L-lysine fails to accumulate in the nucleus. Interestingly, this fragment and the 12 000 mol. wt. tail fragment of nucleoplasmin are both very hydrophilic raising the possibility that cytoplasmic exclusion previously used to explain the nuclear-cytoplasmic distribution of small molecules (Horowitz and Paine, 1976) may be an element in the mechanism by which proteins are accumulated in the nucleus.

Other fragments of the molecule are unstable in the oocyte. It is not possible for us to say whether these fragments could accumulate in the nucleus if they were more stable. The instability of these fragments introduces possible artefacts into the study of their accumulation in the nucleus. The fragments are more stable in the nucleus of the oocyte than in the cytoplasm so that after a time there is some residual material in the nucleus but none in the cytoplasm. This gives the appearance of significant accumulation in the nucleus.

Similar observations have been made by Yamaizumi *et al.* (1978). These authors microinjected non-histone chromosomal proteins into Ehrlich ascites tumour cells by red cell fusion. They observed rapid equilibration between the nucleus and cytoplasm followed by preferential degradation in the cytoplasm. When bovine serum albumin (BSA) was injected it did not equilibrate rapidly and was apparently degraded in both the nucleus and cytoplasm. Therefore the non-histone chromosomal proteins behave in an analogous manner to the H1 fragments NG-H1 and GC-H1 while BSA behaves in an analogous manner to the H1 fragment G-H1.

The rapid selective degradation of abnormal or miscompartmentalised proteins is a well documented phenomenon both for ooocytes (Lane *et al.*, 1979; Colman *et al.*, 1981) and for tissue culture cells (Capecchi *et al.*, 1974; Bigelow *et al.*, 1981; McGarry *et al.*, 1983). This may present problems in attempts to define more precisely the protein features responsible for nuclear migration and accumulation by methods which assay the ability of chimeric molecules to accumulate in the nucleus, notably those which use fluorescent antisera.

While localised degradation limits the interpretation of the results obtained with the unstable fragments it does not affect the conclusion that the carboxy-terminal domain of histone H1 accumulates in the nucleus. The overall properties of this fragment contrast with those of the tail fragment of nucleoplasmin which also accumulates in the nucleus. In addition it has a remarkably simple amino acid sequence; of the 89 amino acids in this fragment, lysine, proline and alanine constitute ~75 of them. Since the other 14 amino acids are scattered, not clustered, these three amino acids must presumably predominate in any sequence which specifies accumulation of this fragment in the nucleus. Lanford and Butel (1984) have isolated and characterised an SV40 mutant in which the large T-antigen fails to accumulate in the nucleus. In this mutant a lysine residue is replaced by an asparagine residue. This lysine residue is one of four in a short sequence which is bounded at each end by proline residues. Also the amino acids lysine and proline are present in a sequence which lies in the amino-terminal 13 residues of the yeast $\alpha 2$ protein, which when linked to β-galactosidase cause it to accumulate in the yeast nucleus (Hall *et al.*, 1984). These authors found closely homologous sequences to the $\alpha 2$ sequence in $\alpha 1$ and in the histones H4 and H2B. The amino acids lysine, proline and alanine are conspicuous in these short regions of homology. A homologous region in the

histone H2A lacked alanine and lysine but contained isoleucine and arginine.

The results we describe here are completely consistent with these findings and illustrate that, at least for the histones, the sequence requirements for accumulation in the nucleus may be simple.

Materials and methods

Purification of calf thymus histone H1 and the proteolytic fragments

Each of the pepetides used in this study was prepared from total calf thymus H1 which had been isolated by 5% perchloric acid extraction (Johns, 1976) and purified by chromatography on Amberlite CG-50. The peptides NG-H1 (residues 1 – 121) and C-H1 (residues 122 – 210) were purified from a thrombin digest by chromatography on carboxymethyl cellulose (Whatman CM-32) (Chapman *et al.*, 1976). The globular domain, G-H1 (residues 36 – 121), was isolated from a trypsin digest by chromatography on Sephadex G-50 (Hartman *et al.*, 1977). The peptide CG-H1 (residues 33 – 210) was a gift from Dr. L. Bohm and had been prepared by purification of a submaxillary digest on Bio-Gel P-60 and carboxymethyl cellulose (Whatman CM-52) (Bohm *et al.*, 1982).

Labelling with Bolton and Hunter reagent

N-Succinimidyl 3-(4-hydroxy 5-[^{125}I]iodophenyl) propionate was obtained from Amersham International PLC. The procedure of Bolton and Hunter (1973) was modified to reduce the level of protein modification. Routinely 200 μCi of reagent (sp. act. 2000 Ci/mmol) was reacted with 10 μg of protein in 10 μl of 100 mM sodium borate pH 8.5, 50 mM NaCl on ice for 30 min. The reaction was terminated by adding 10 μl 1 M glycine, 1 μl 10 mg/ml potassium iodide, 1 μl of 0.5% phenol red and 30 μl column buffer. The sample was then passed down a 1.5 ml (20 cm x 0.3 cm) column of Sephadex G-25 equilibrated with column buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA 0.2% gelatin). The equilibrated column was pre-washed with 50 μl 1 mg/ml lysine. Fractions (0.2 ml) were collected and counted with a Beckman Bio Gamma counter. Phenylmethylsulfonyl fluoride (PMSF) was added to the labelled protein to a concentration of 0.1 mM to inhibit proteolysis. The phenol red marked the included volume and co-eluted with non-incorporated reagent.

In the case of poly-L-lysine homopolymer the peak of activity from the G-25 column was dialysed overnight at 4°C against 3000 volumes of 50 ml Tris-HCl pH 7.5 50 mM NaCl 0.1 mM EDTA to ensure complete removal of all non-incorporated reagent.

Microinjection and culture of oocytes from X. laevis

Ovaries were dissected from female *X. laevis* and maintained in modified Barths saline at 20°C as described by Gurdon (1976). Each fully grown oocyte recieved injections of ~50 nl aimed away from the centre of the oocyte and towards the yolky vegetal pole to avoid injecting into the nucleus.

Isolation of nuclei and cytoplasms from oocytes

Oocyte nuclei were removed manually by gently tearing the animal pole of injected oocytes with two pairs of watchmaker's forceps. Isolated nuclei were transferred by micropipette to 95% ethanol on ice for storage during collection. Enucleated cytoplasms were collected in the minimum volume of saline (1 – 2 μl) and frozen on solid CO_2 in siliconised glass tubes during collection. The accumulated samples from an experiment were stored at −80°C until analysed.

Analysis of separated nuclei and cytoplasms

Oocyte nuclei in 95% ethanol were counted and dried *in vacuo* and taken up in gel sample buffer. Oocyte cytoplasms were counted and homogenised by vortexing in 50 μl of 15 mM Tris HCl pH 6.8, 1 mM PMSF for 1 min. The homogenate was then extracted with an equal volume of 1,1,2-trichloro-trifluoroethane. The supernatant was collected and counted. The recovery of radioactivity for nuclei and cytoplasm was used to adjust the peak areas from the densitometer tracers of the autoradiographs.

Volumes equivalent to equal numbers of nuclei and cytoplasm were analysed by SDS-gel electrophoresis according to the method of Blattner *et al.* (1972). After electrophoresis the gels were dried and quantitative autoradiography was carried out with a pre-flashed film at −70°C and an intensifying screen (Laskey and Mills, 1977). Densitometry of the autoradiographs was performed with a Joyce-Loebl scanning microdensitometer.

In order to obtain satisfactory electrophoresis of radiolabelled histone H1 and the proteolytic fragments, 10 μg cold carrier H1 was added to each sample either at the beginning of the extraction procedure or immediately before gel electrophoresis.

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