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DNA injections into *Xenopus* embryos: fate of injected DNA in relation to formation of embryonic nuclei

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INTRODUCTION

Microinjection experiments using cloned gene templates into fertilized eggs of *Xenopus laevis* provide an interesting experimental system to study factors involved in control of gene expression, as well as possible mechanisms of gene integration and rearrangements of injected DNA templates into the *Xenopus* genome. In addition, for many types of cloned genes it is possible to compare transcription characteristics obtained from an embryo injection experiment with results from gene-injected oocytes.

In the case of DNA injection experiments into *Xenopus* oocytes, systematic studies have been carried out on the stability and chromatin configuration of injected DNA following injection into the cytoplasm or into the nucleus ('germinal vesicle') of the large *Xenopus* oocyte. It was found that DNA can be injected into both cellular compartments, but that injected DNA is rapidly degraded after injection into the cytoplasm, whereas DNA injected into the nucleus of the oocyte is not degraded but assembled into chromatin (Wyllie, Laskey, Finch & Gurdon, 1978; Laskey, Gurdon & Trendelenburg, 1979).

By contrast, in a typical embryo injection experiment, the DNA sample is nearly always deposited somewhere in the cytoplasmic region of the animal half of a symmetrized *Xenopus* egg or an early 2-cell stage. From early investigations onwards, it was known that embryo-injected DNA is not only stable in the cytoplasm but is also replicated (Gurdon, 1974*a*; for a recent review on chromatin replication during embryogenesis see Laskey, 1985).

Whereas a rapidly increasing number of investigations is concerned with the biochemical and molecular analysis of the conformation and persistence of embryo-injected DNA (see below, and reviews by Gurdon & Melton, 1981; Etkin

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& DiBerardino, 1983; Etkin, Pearman, Roberts & Bektesh, 1984), relatively little is known of the ultrastructural localization of injected DNA samples. In a recent study, Forbes, Kirschner & Newport (1983) investigated structural aspects of injected DNA using microinjection of bacteriophage λ-DNA into unfertilized *Xenopus* eggs. It was shown that injected λ-DNA apparently triggers the formation of nuclear envelopes around small droplets of injected DNA. Microscopically such structures can be recognized as 'pseudonuclei' (Forbes *et al.* 1983).

These findings stimulated us to start a combined biochemical and structural analysis concerning the conformation of injected DNA and to characterize the ultrastructural aspects of embryo-injected DNA samples.

PERSISTENCE AND CONFIGURATION OF INJECTED DNA DURING EARLY EMBRYOGENESIS

In most examples analysed so far, DNA was injected into fertilized eggs in the form of supercoiled circular DNA. The persistence and possible replication of injected circular DNA can thus be conveniently analysed by characterization of amounts of circular DNA at defined stages of early embryogenesis. In systematic studies of the major injection parameters it was first established that only limited amounts of DNA can be injected into embryos (e.g. when compared to amounts injectable into oocyte nuclei) in order to allow development of sufficient numbers of injected embryos (Gurdon & Brown, 1977; Bendig, 1981; Rusconi & Schaffner, 1981). Results from several embryo injection studies using different templates led to the conclusion that circular DNA persists and eventually may be amplified through early embryogenesis, but becomes degraded after the gastrula stage. However, it was also found that a small number of injected gene copies was integrated into the *Xenopus* genome and it was shown that such integrated copies can persist up to the adult stage (Rusconi & Schaffner, 1981; Andres, Muellener & Ryffel, 1984; Rusconi, 1985). Since most studies used heterologous gene templates for injection, several possibilities for the observed differences in replication and integration could be discussed (see Méchali & Kearsey (1984) for discussion on sequence requirements for DNA replication in *Xenopus* eggs). It was only recently that embryo-specific genes could be characterized and analysed for their persistence after injection into fertilized eggs. Interestingly it could be shown that a gastrula-specific clone is not amplified during early embryogenesis and persists as extrachromosomal DNA up to the tailbud stage. In this case, no integration of injected gene copies into the embryo genome could be detected (Krieg & Melton, 1985). To test our injection parameters in detail, we injected a chicken ovalbumin gene into fertilized *Xenopus* eggs and analysed the fate of injected DNA before and after gastrulation. As shown in Fig. 1A,B, presence of extrachromosomal circular DNA could be demonstrated at early cleavage stages. In contrast, DNA extraction from neurula stages showed that extrachromosomal gene copies were no longer present, but a small number of gene copies were found to be integrated into the *Xenopus* genome. Integrated copies persist up to the swimming tadpole

stage. Preliminary results indicate that the integrated copies were complete and not rearranged. In addition, we observed a transient, low level transcription at cleavage stages 8 to 11 of injected embryos in contrast to results from oocyte injection experiments, where no significant amounts of ovalbumin RNA could be detected (Trendelenburg, Mathis & Oudet, 1980; Trendelenburg, 1983).

FORMATION OF THE NUCLEOCYTOPLASMIC ARCHITECTURE DURING EARLY CLEAVAGE

Studies on the ultrastructural localization of injected DNA require a detailed knowledge of the structural organization of embryonic nuclei and the surrounding cytoplasm during early cleavage stages. Most structural information up to now was obtained from the analysis of thick sections through paraffin-wax-embedded embryos. The published electron microscopic studies on early embryo nuclear structures are on rather specialized topics. Among these studies are (i) a detailed

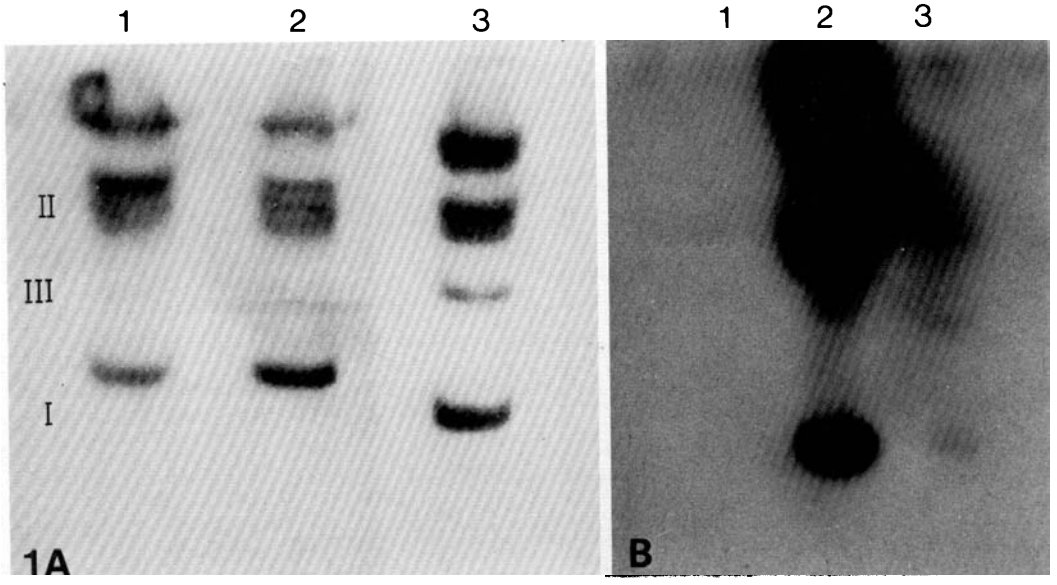

Fig. 1. Results of a DNA injection experiment into fertilized *Xenopus* eggs using a cloned chicken ovalbumin gene (for details of the clone see Trendelenburg *et al.* 1980). Fertilized *Xenopus* eggs were obtained as described by Billet & Wild (1975). Immediately after the rotation reaction, eggs were briefly irradiated under a u.v. lamp to soften the jelly coat (for details see Gurdon, 1977). Fertilized eggs up to early two-cell stages were injected with 100 to 150 pg of DNA. DNA extraction was done according to Rusconi & Schaffner (1981). Samples were run on 0·8% agarose gels (A) or 1% agarose gels (B) and hybridized against ^{32}P-labelled ov cDNA. Circular DNA can be extracted from early embryos (cleavage 8, line 1; cl. 11, line 2; marker DNA, line 3; I super-coiled circular DNA, II nicked circular DNA, III linear DNA). From neurula stage embryos (Nieuwkoop & Faber, stage 23, Fig. 1B) no significant amounts of circular DNA could be extracted. Total DNA was extracted, cut with EcoRI, and analysed as indicated above (control: noninjected neurulae, line 1; marker ov cDNA, line 2; injected neurulae, line 3).

analysis on the dorsally located conspicuous yolk-free cytoplasmic zone which was found to be a characteristic of the symmetrized egg approximately 60 min post-fertilization. It was shown that in the still uncleaved egg a relatively large yolk-free zone exists in the dorsoanimal quadrant of the egg (Herkovits & Ubbels, 1979). The EM data showed that this zone is particularly rich in cytoplasmic vesicles and mitochondria. The area is characterized by the absence of large yolk particles (longitudinal diameter of this zone is 100–150 μm). (ii) In another EM study, chromatin organization within embryonic nuclei of blastula and gastrula stages was compared (Csaba & Do, 1974).

To allow analysis of the nucleocytoplasmic architecture at high magnification, we decided to use sections through epon-embedded material. This approach has the advantage that well-fixed embryos can be sectioned at a precise thickness, normally 2–3 μm, but thinner sections of large areas can also be obtained, if necessary, for optical as well as electron microscopic analysis. Sections of this kind allow light microscopic analysis using phase or interference contrast microscopy of unstained specimens at high magnification, in particular if a conventional light microscope is equipped with a system for video-enhanced contrast (see below). An overview of the typical nuclear structures seen in sections of early embryos is shown in Fig. 2. In most cases, typical interphase nuclei are seen and screening for the presence of interphase nuclei can be done rapidly at low magnification. If no interphase nuclei are seen at low magnification typical aspects of metaphase chromosomes can be recognized using phase contrast at high magnification (Fig. 2B,C; for direct comparison of dimensions, Fig. 2A–C is enlarged to the same scale). In addition, it was found that all nuclear structures (interphase nuclei as well as metaphase plates) are typically located in the central area of a large, predominantly yolk-free cytoplasmic zone. Such cytoplasmic regions are very large during the earliest cleavage cycles and become progressively smaller as cleavage proceeds. A comparison of such areas is shown in Fig. 3A (cleavage cycle 2) and Fig. 3B (cl. 7), again both micrographs are enlarged to the same scale. Comparison of Fig. 3A and B indicates in addition that, whereas the size of the cytoplasmic area decreases drastically, the mean diameter of embryonic nuclei does not change significantly during these early cleavages. This observation is substantiated by the dimensions and structural aspects of interphase nuclei of cleavages 2, 4 and 6, as shown in Fig. 4A–C.

As shown in Fig. 4A–C, nuclei are characterized by large diameters, e.g. up to 30 μm, and the presence of intranuclear membrane folds. These observations are in line with earlier observations from *in situ* fixed embryonic nuclei (Hay & Gurdon, 1967; Bonnano, cited in Gerhart, 1980; and measurements on isolated embryonic nuclei, Farzaneh & Pearson, 1978). Details of the size distributions of mean diameters for interphase nuclei as well as for the corresponding yolk-free cytoplasmic zones where the nuclei were found to be located are shown in the diagram of Fig. 5. Whereas a drastic size reduction of mean diameters for cytoplasmic zones was observed from cleavage 2 to cleavage 10, a reduction of

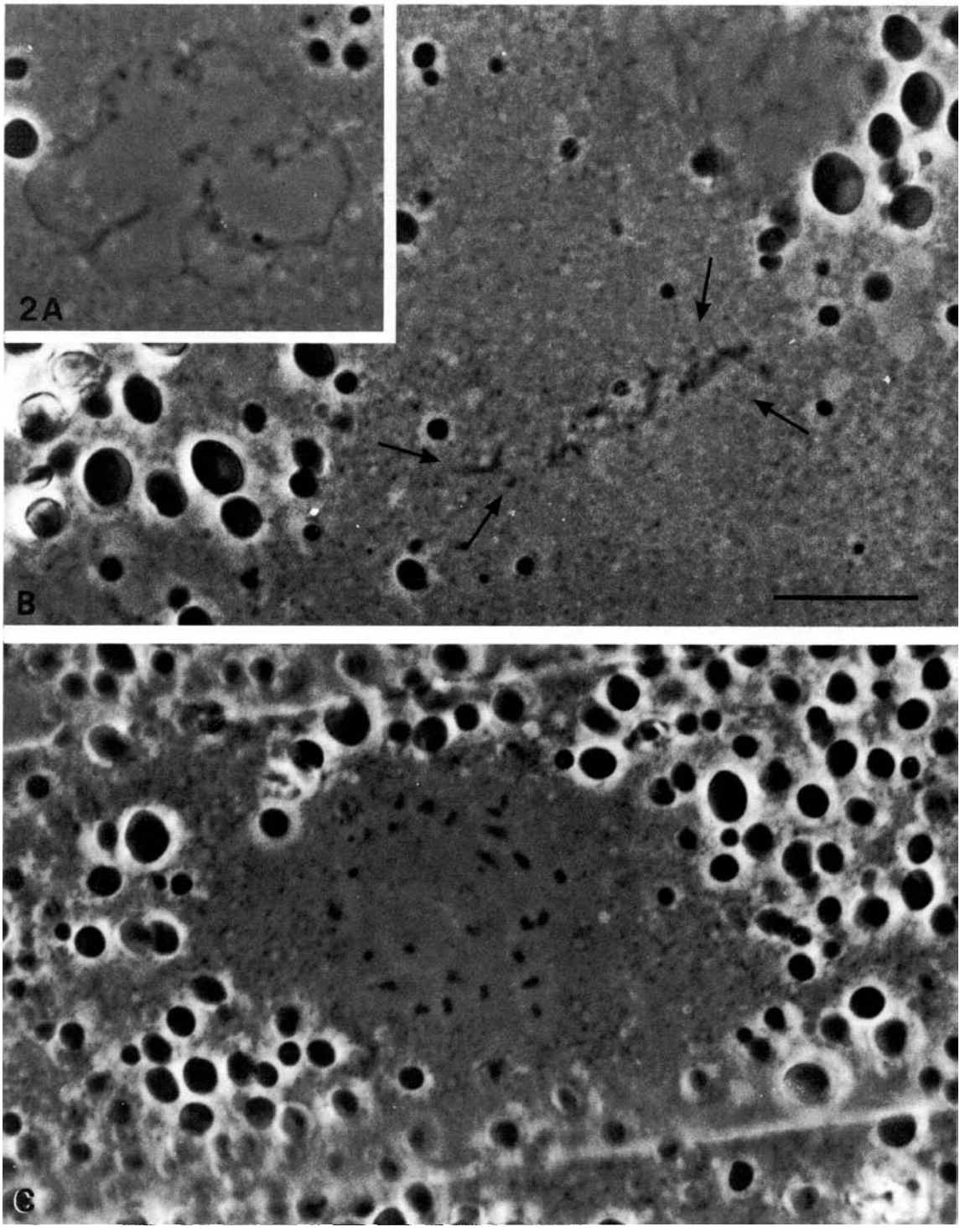

Fig. 2. Light microscopic analysis of 3 μm sections through Epon-embedded early cleavage stages. Nuclear structures are typically identified in phase contrast as two predominant types. (A) Interphase cell nucleus from a cleavage 2 embryo. (B) Transverse section through an early metaphase plate of a cleavage 5 embryo. (C) Tangential section through a late metaphase of a cleavage 3 embryo. For methodological details see legend of Fig. 3. Bar, 10 μm.

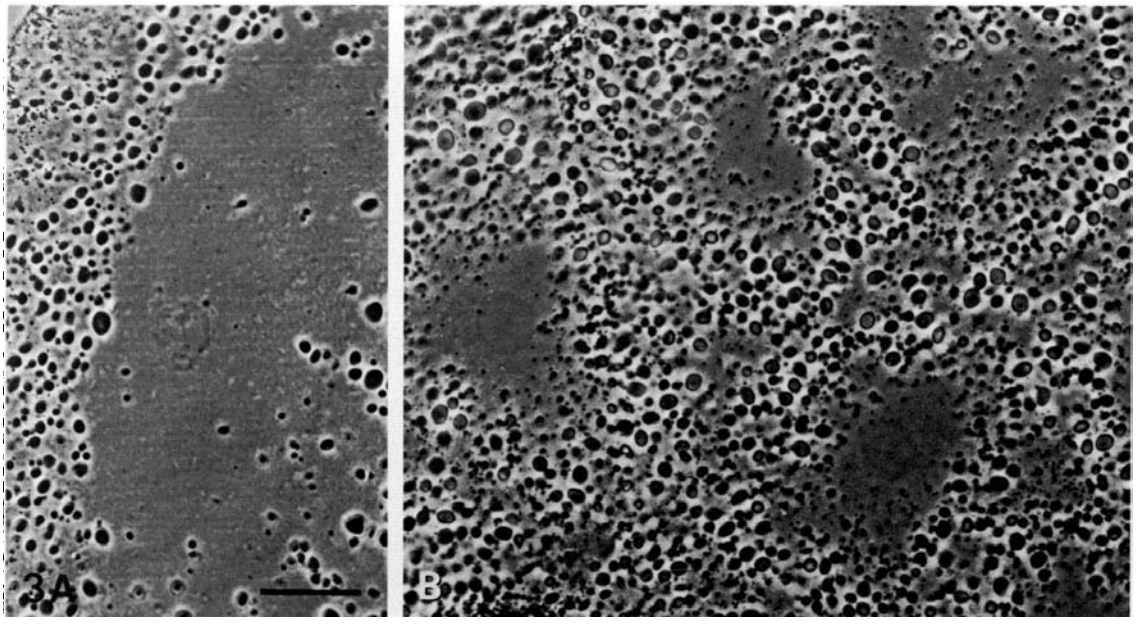

Fig. 3. Survey micrographs on nucleocytoplasmic architecture of early *Xenopus* embryos (A, cleavage 2; B, cleavage 7). Note that diameters of interphase nuclei are similar for the two stages shown. By contrast, the areas of surrounding yolk-free cytoplasmic zones are strikingly different in size. Fertilized eggs were obtained as described in the legend of Fig. 1. Embryos were fixed and embedded for electron microscopy as described by Kalt & Tandler (1971). Sections (2–3 μm thick) were examined using phase contrast light microscopy for the presence of embryonic nuclei and/or yolk-free cytoplasmic zones. Bar, 30 μm.

mean nuclear diameters was observed during cleavage cycles 11 to 14. Nuclear diameters are then around 10 μm and remain almost constant during the successive later stages of development (e.g. at stages 14 and 20 according to the staging system of Nieuwkoop & Faber, 1967; see Fig. 5). For comparison, first appearances of nuclear lamins L_I and L_{II} are indicated in Fig. 5 (for details see Stick & Hausen, 1985; for localization studies on embryonic nuclei see Benavente, Krohne & Franke, 1985). The way in which this complex nuclear architecture is achieved is not yet established. Work is in progress to apply video-enhanced contrast light microscopy (for details see Weiss, 1986) to thick sections of embryonic nuclei. Using this methodology, different focus levels can be analysed for the presence and spatial orientation of intranuclear membranes (Fig. 6A,B), information which is difficult to obtain from thin-sectioned nuclei using electron microscopy (Fig. 6C).

In conclusion, interesting changes in nucleocytoplasmic compartmentalization can be observed from thick-sectioned material. It is clear, however, that additional studies are required for a more close correlation of the observed structural changes with the cell cycle data (Newport & Kirschner, 1982; for recent review see Satoh, 1985).

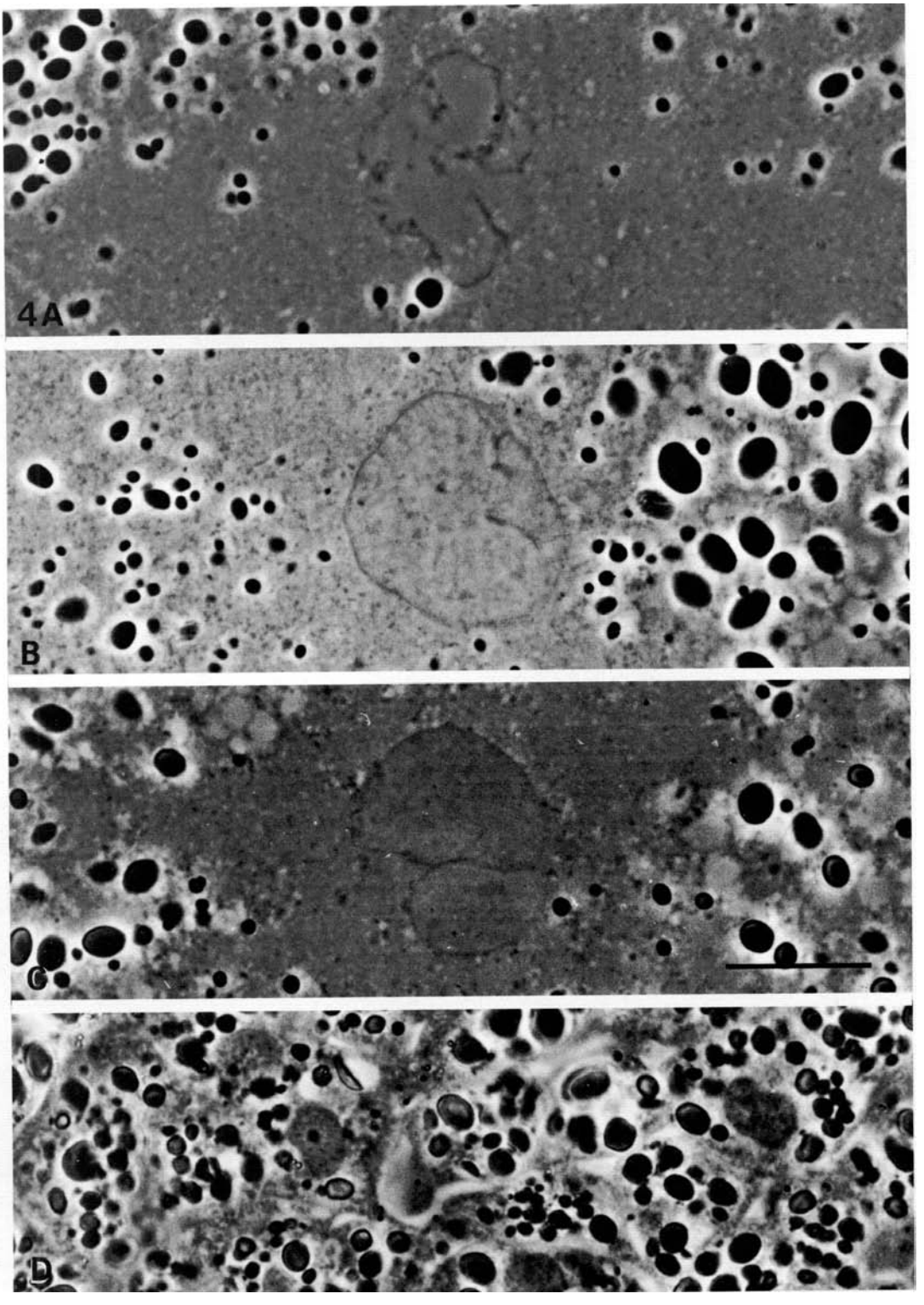

Fig. 4. Comparison of size and structure of embryonic nuclei during early cleavage. (A) Cleavage 2; (B) cleavage 4; (C) cleavage 6; (D) cleavage 12. Embryonic nuclei that are irregular in shape are characterized by their large size and the presence of conspicuous intranuclear membrane folds. Bar, 20 μm.

RECOGNITION OF 'PSEUDONUCLEI' IN EMBRYOS FOLLOWING INJECTION OF BACTERIOPHAGE λ-DNA

For structural studies on embryo-injected DNA we chose injection experiments of bacteriophage λ-DNA for the following reasons.

(i) In a detailed investigation Forbes *et al.* 1983 showed that λ-DNA is a good template for induction of membrane structures around injected DNA, if the DNA was injected into unfertilized eggs. It was observed that, 1·5 to 2 h following injection into the unfertilized egg, λ-DNA is predominantly contained in nuclear structures approximately 6 μm in diameter (range 1–20 μm).

(ii) Using electron microscopy it was shown that the DNA samples were surrounded by a typical nuclear membrane structure (Forbes *et al.* 1983).

For our structural investigations we injected 2–5 ng of λ-DNA into fertilized eggs. Special care was taken to perform the experiment exclusively on batches of fertilized eggs that exhibited a particularly high synchrony of cleavages. In agreement with results from earlier investigations using injection of relatively high amounts of DNA (Gurdon & Brown, 1977; Rusconi & Schaffner, 1981) no significant percentage of embryos was noted that were arrested during early

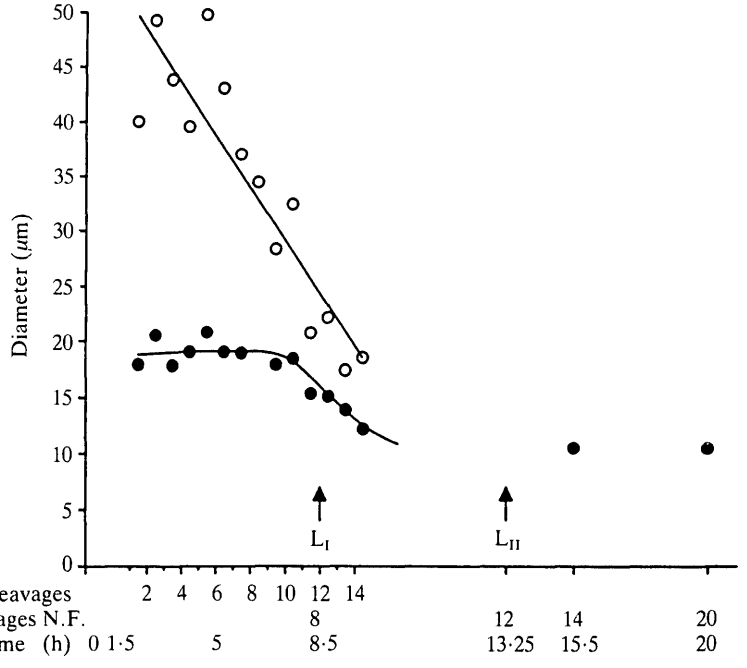

Fig. 5. Correlation of mean diameters of yolk-free cytoplasmic zones (○) with mean diameters of embryonic nuclei (●) of successive early cleavage stages. L_I and L_{II} denote the appearance of lamins L_I and L_{II} during early cleavage (data from Stick & Hausen, 1985). Cleavage stages are characterized as cleavage cycles according to Newport & Kirschner (1982), as stages (N.F.) according to Nieuwkoop & Faber (1967) and time (h postfertilization). Each point represents a mean value consisting of 5–12 individual measurements. Calculation of mean diameters was done using the formula a+b/2 where a represents the length diameter, and b the diameter in width, perpendicular to a.

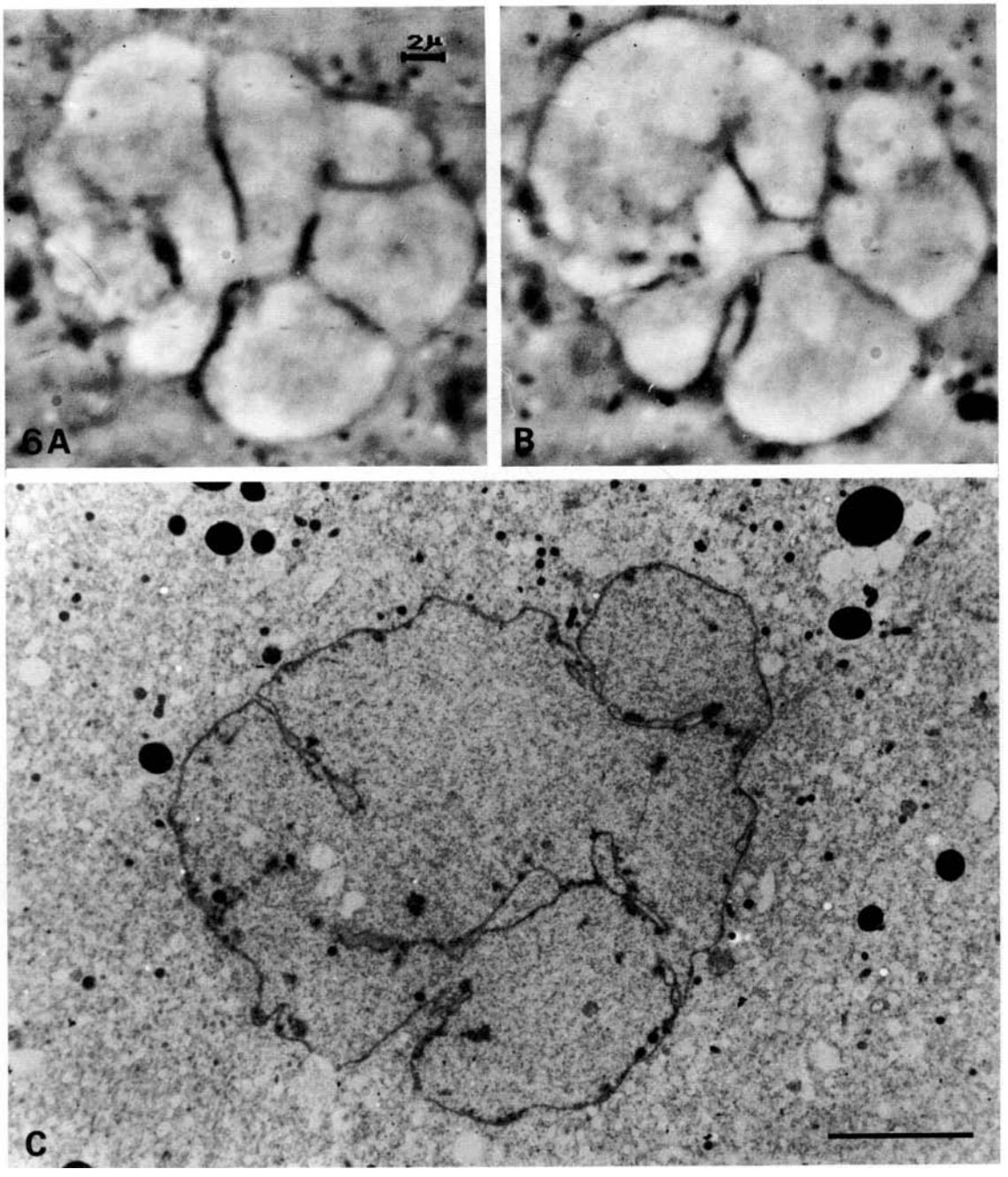

Fig. 6. Ultrastructural aspect of embryonic nuclei at high magnification. Two different focus levels through a 3 μm thick section of an interphase nucleus of a cleavage 7 embryo (phase contrast, video-enhanced contrast light microscopy). Focus level of B is 2 μm below focus level of A. Note the high complexity of membrane folds. The typical electron microscopic aspect as seen in thin-sectioned nuclei is seen in C (example shown, cleavage 2 embryo). Numerous intranuclear membranes can be clearly identified. Bar in C, 5 μm.

cleavage. In fact, in good batches of fertilized injected eggs, as many as 60–80 % of injected embryos reached cleavage cycle 10 as compared to controls. Embryos were fixed during early cleavage cycles, and thick sections analysed for the

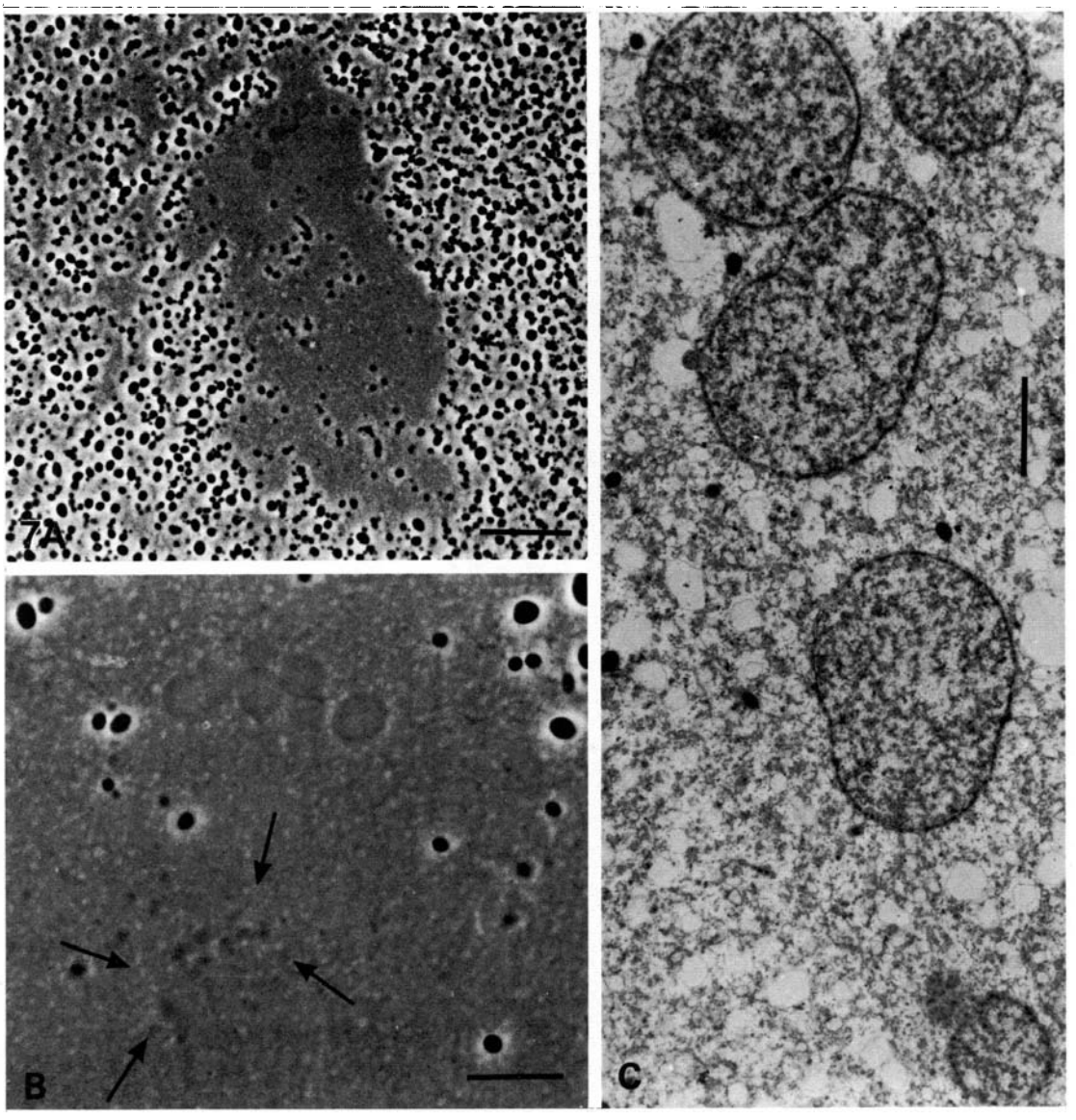

Fig. 7. Recognition of 'pseudonuclei' from bacteriophage λ-DNA injected *Xenopus* embryos. Fertilized eggs were injected with 2–5 ng. λ-DNA as described in the legend of Fig. 1. Developing embryos were fixed and embedded for electron microscopy as described in the legend of Fig. 3. (A,B) Phase contrast light micrographs through cytoplasmic zones of cleavage 5 embryos. (A) Small nuclear structures are seen at a marginal position of the cytoplasmic area. (B) The cluster of small nuclei is located in close proximity to a metaphase plate of embryonic chromosomes. (C) Ultrastructural details of organization of 'pseudonuclei' (cleavage 5 embryo). A, 30 μm; B, 10 μm; C, 2 μm.

presence of small 'pseudonuclei' in the large, yolk-free cytoplasmic zones of early embryonic cells (see preceding section for details). Under the light microscopic screening conditions used (phase contrast microscopy of 3 μm sections) nuclear structures with diameters of 3 μm and more should be visible in the yolk-free cytoplasmic zones. Yolk-free cytoplasmic zones were analysed using serial sections from 30 embryos. Morphological evidence for the presence of bacteriophage DNA-derived nuclear structures within these cytoplasmic areas was obtained for 30 % of injected embryos. In most cases 'pseudonuclei' were observed to occur as clusters of small nuclear structures, approximately 4–8 μm in diameter, which were found to be typically located at the periphery of individual yolk-free zones (Fig. 7A, cleavage 5 embryo). From their ultrastructure (Fig. 7C) these structures resemble very closely the nuclear structures seen in bacteriophage DNA-injected unfertilized eggs (cf. figs 2, 3, 5 in Forbes *et al.* 1983). An interesting result of our investigation was the observation that, in all cytoplasmic zones where we observed typical 'pseudonuclei', no coexistence of 'pseudonuclei' next to a typical large interphase nucleus was seen. In this regard, two types of results were obtained.

(i) Embryos fixed at cleavage cycles 2, 3 and 4 following DNA injection showed the presence of up to 10 closely associated 'pseudonuclei' in up to two yolk-free cytoplasmic zones per individual embryo. In most cases observed, 'pseudonuclei' were arranged as a straight line at the periphery of the cytoplasmic zone. Where no 'pseudonuclei' were found in this class of embryos, the cytoplasmic zones showed the presence of highly reticulated embryonic nuclei.

(ii) The second group of embryos was characterized as follows. Apparently, these embryos were fixed at metaphase, since in almost all of their cytoplasmic zones typical metaphase plates could be observed (cleavages 3/4, 4/5, 6/7; for typical aspect of metaphase plates in noninjected embryos see Fig. 2B,C). In the cytoplasmic zones of these embryos, where 'pseudonuclei' were detected, metaphase plates of embryonic chromosomes were also seen (Fig. 7B). Work is in progress to use this approach in combination with autoradiography (Gurdon & Brown, 1977), DNA-staining techniques (Forbes *et al.* 1983) and scanning and transmission electron microscopy (Tröster *et al.* 1985) to analyse the interaction of injected λ-DNA with the embryonic nuclei during interphase and metaphase in more detail.

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