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Technical Article

Selecting karyophilic DNA cis elements in *Xenopus laevis* oocytes; a new approach

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ABSTRACT The intracellular localisation and mobility of exogenous DNA introduced into *Xenopus laevis* oocytes is largely unknown. In this paper, we report a new technique to investigate the cytoplasmic/nuclear transport of a random pool of linear, double-stranded, oligomeric DNA of 147 bp in length. We chose a combinatorial approach which made use of repetitive rounds of selection and amplification to search for new cis elements mediating nuclear import or retention. A new PCR-based methodology was established to reliably detect exogenous DNA in subcellular and total extracts prepared from *Xenopus laevis* oocytes. Studies *in vivo* and with cellular extracts indicate the presence of a highly efficient nuclease activity in the nuclear compartment. The described combinatorial approach constitutes a promising tool for the isolation of novel DNA cis elements which may play an important role in the nuclear internalisation and retention of exogenous DNA in *Xenopus laevis* oocytes.

KEY WORDS: *double-stranded DNA, intracellular transport, Xenopus laevis oocytes*

Introduction

In naturally occurring biological systems, the nuclear import of DNA is particularly relevant when considering viral infection processes. Similarly, the delivery of recombinant DNA to cells and its nuclear transcription are crucial events in molecular biology and molecular medicine. In mammalian cells, less than 1% of DNA molecules which are injected into the cytoplasm of cells are accessible for active gene expression (Dowty *et al*., 1995). In the present study, we examined the possibility that recombinant DNA can be imported into the nucleus via specific DNA sequences. This possibility is supported by recent findings which suggest that DNA cis elements may signal and enhance this process (Dean, 1997; Längle-Rouault *et al*., 1998; Vacik *et al*., 1999). Since transcription factors can presumably bind to specific sequences of DNA delivered into the cytoplasm (Dean, 1997) and many of these factors contain nuclear localisation signals (NLSs), complexed DNA may thus coat itself with nuclear import signals. The NLSs present in the nucleoprotein complex could then interact with specific importin receptors and enter the nucleus via normal nuclear import machinery (Nigg, 1997). This hypothesis is supported by the finding that increased nuclear import of naked adenoviral DNA in monkey cells (Greber and Kasamatsu, 1996) and EBV (Epstein-Barr virus)-derived double stranded DNA in human cells (Längle-Rouault *et al*., 1998) apparently occurs via DNA cis elements. While these eukaryotic systems are of obvious direct importance, studies on the nuclear import of nucleic acids have also been successfully performed using *Xenopus* oocytes (Guralnick *et al*., 1996; Grimm *et al*., 1997; Rutjes *et al*., 2001). Because of their status as non-dividing cells, *Xenopus* oocytes were chosen as an appropriate *in vivo* test system to establish a protocol for a combinatorial search for nuclear uptake and retention signals.

In this paper, we describe a methodology which is suitable for the cytoplasmic and nuclear delivery of exogenous DNA into *Xenopus* oocytes and for the determination of mechanisms mediating its retention within, and exchange between, these compartments. PCR-based techniques allowed us to detect the presence of exogenous DNA in both the nucleus and cytoplasm of oocytes. This clearly represents an advance over previous studies (Maryon

Abbreviations used in this paper: bp, base pair(s); DS, dyad symmetry element; EBNA1, Epstein-Barr nuclear antigen-1; EBV, Epstein-Barr virus; FR, family of repeats; NLS, nuclear localisation signal; oriP, origin of replication; PCR, polymerase chain reaction; TCA, trichloroacetic acid.

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and Carroll, 1989; Gurdon and Melton, 1981; Colman, 1984) which employed lower sensitivity techniques, such as Southern hybridisation or visualisation of ethidium bromide-stained DNA bands in agarose, to detect the total degradation of injected nucleic acid. The novel method which we report here can be developed to select for improved transport of DNA from the cytoplasm to the nucleus and for increased retention of DNA in the nucleus.

Experimental Protocols

Injected DNA

Two different kinds of DNA were introduced into oocytes: a 5.6 kbp long plasmidic supercoiled DNA (pTG 11220, Fig. 1A) and an oligomeric double-stranded DNA fragment consisting of two primer binding sites flanking a 100 bp long randomised central element, with a total length of 147 bp (Fig. 1B). Each oocyte cytoplasm and nucleus was microinjected with 50 nl and 20 nl, respectively, of a solution of 100 ng/µl DNA, corresponding to 5 ng and 2 ng of DNA per cytoplasm and nucleus, respectively. The pool of random DNA (TIB MOLBIOL; Berlin, Germany) has a theoretical complexity of approx. 10^{17}, which is reduced to approx. 10^{11} when considering the concentration and volume of DNA injected into the oocytes. The pool was purchased as single-stranded DNA and amplified by preparative PCR using the specific primers PA and PB. The pTG 11220 plasmid (Fig. 1A) harbours Epstein-Barr virus (EBV) oriP sequences, and was generously donated by Transgene S.A. (Strasbourg, France). The whole oriP segment is composed of the following two functional subelements (Reisman *et al.*, 1985; Yates *et al.*, 1985): the family of repeats (FR) comprising 20 copies of a 30 bp binding site motif for the viral protein EBNA1 and, 960 bp downstream, the dyad symmetry element (DS), which consists of four copies of a 16 bp binding site for the EBNA1 protein. The DS region is the site for initiation of replication (Gahn and Schildkraut, 1989; Wysokenski and Yates, 1989), whereas the FR element acts as an EBNA1-dependent enhancer participating in DNA replication. The pTG 11220 plasmid contains the total oriP region of replication, with a deletion of the DS elements (oriP ΔDS position 8994 to 9133 according to Baer *et al.*, 1984). The oriP ΔDS fragment has previously been shown to mediate DNA transport into the nucleus (Längle-Rouault *et al.*, 1998).

Oocyte Recovery from Xenopus laevis

Adult *Xenopus laevis* females were anaesthetised in a solution of 0.1 % MS222 (tricaine methanesulfonate; SIGMA, Munich, Germany). Part of the ovaries of the animals was removed through a 1 cm long cut in the ventral region. The incision was then sewn up and the frog was laid in a small amount of water until awakening (Hames and Higgins, 1984). The whole procedure was repeated with a time interval of two months. A total of 19 animals were used to provide oocytes for this study and each of them was identified by branding (Kay and Peng, 1991). Recovered oocytes were kept at 19°C for a few hours in Barth's medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM Ca $(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 mM HEPES pH 7.6; Gurdon and Wakefield, 1986). Individual ovaries were divided in groups of 2 to 4 oocytes with the help of a pair of small tweezers (Dumont No. 5) for injections (Dumont, 1972).

Injection of Oocytes and Isolation of Nuclear and Cytoplasmic Fractions

Stage VI (Nieuwkoop and Faber, 1967) fully grown oocytes were used for experiments. They were directly used without defolliculation, i.e. they were not deprived of the first layer of somatic cells surrounding the oocyte. Groups of 10 oocytes were pooled for each single experiment of DNA injection into a specific subcellular compartment, as this number was found to be the minimum required to guarantee satisfactory reproducibility. In order to inject oocytes, the so-called "blind method" was used

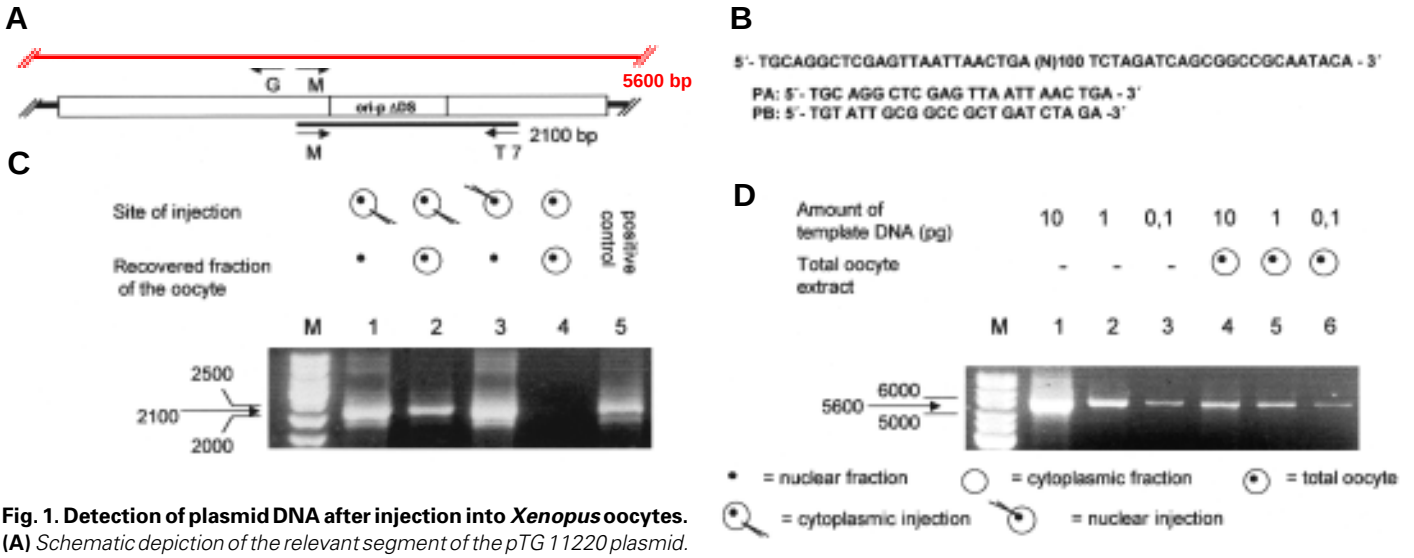

Fig. 1. Detection of plasmid DNA after injection into *Xenopus* oocytes. **(A)** *Schematic depiction of the relevant segment of the pTG 11220 plasmid. The size of the amplicon generated using the T7 and M primers is 2100 bp (black bar) and 5600 bp when the G and M primers are employed (red bar).* **(B)** *Nucleotide sequences of a randomised pool of double-stranded DNA which can be amplified by primers PA and PB. The length of the DNA in the pool is 147 bp.* **(C)** *Detection of PCR products after site-specific injection of plasmid DNA into oocytes. Lane 1: detection of plasmid DNA in nuclear extracts subsequent to its injection into the cytoplasm. Non-specific amplification of oocyte genomic DNA was not observed (negative control, lane 4). Lane 5: positive control for PCR (31 cycles) with 100 pg of the plasmid as template.* **(D)** *Influence of oocyte extract on the yield of PCR amplification. Indicated amounts of the pTG11220 template were amplified using the G and M primer pair in the absence (lanes 1-3) or presence of 10 µl of oocyte extract (lanes 4-6).*

(Gurdon, 1976; Colman, 1984). The cytoplasm was injected with 50-60 nl of a DNA solution (100 ng/µl), whereas a maximum of 20 nl was injected into the nucleus, in accordance with its toleration limit (Sive *et al.*, 2000). These injection volumes were taken into account when calibrating the injection capillaries (Gurdon, 1974).

Following injection, the oocytes were incubated at 19°C for 3 hours in isolation buffer, pH 7.2 (83 mM KCl, 17 mM NaCl, 6.5 mM Na_2HPO_4, 3.5 mM KH_2PO_4). Thereafter the injected oocytes were fixed with 10% trichloroacetic acid (TCA) for 10 minutes (Kay and Peng, 1991). This treatment facilitates the subsequent separation of nucleus and cytoplasm. The concentration and time of TCA treatment, which induces protein coagulation, were chosen in order to ensure that the low molecular weight DNA was not damaged. Subsequently, the oocyte material was transferred to small Eppendorf tubes and shock frozen in liquid nitrogen.

Preparation of Oocyte Cell Fractions

Nuclear or cytoplasmic fractions and whole oocytes were frozen in liquid nitrogen and stored at –80°C until DNA isolation was performed. The following protocol represents the optimised guideline used in the experiments, even though in the course of the selection some parameters were varied in order to further optimize the yield of recovered DNA (see Results and Discussion section).

To prepare extracts from subcellular compartments or whole oocytes, 30 µl of homogenising buffer (10 mM Tris HCl, pH 7.5; 1 mM $MgCl_2$; 10 mM NaCl; 2% SDS; 1 mg/ml proteinase K) were added for every oocyte derivative. Two types of homogenisation were performed: for nuclei, simply pipetting up and down was sufficient, whereas for cytoplasmic fractions or whole oocytes, it was necessary to crush the cellular material with a stopped Pasteur pipette until large clumps of cytoplasm had disappeared. Subsequently, proteinase K digestion (final concentration: 1 mg/ml) was carried out at 37°C for 90 minutes followed by a first extraction, which consisted of adding 20 µl 5 M NaCl, 300 µl phenol and 300 µl isoamylalcohol /chloroform (1:24) to every 300 µl of homogenized oocyte extract. The mixture was centrifuged at 5000 g for 15 min and this extraction was repeated three times with equal volumes of phenol /isoamylalcohol chloroform. Finally, low molecular weight DNA was precipitated by adding two volumes of 100% ethanol, followed by centrifugation and washing with 70% EtOH. After the final centrifugation, the precipitate was dried and resuspended with 50 µl of TE (pH 8), and 5 µl were used as template for PCR reactions.

PCR Conditions, Degradation Assays and Native Gel Electrophoresis

Isolated, purified plasmidic DNA was amplified using the following primers:

G: 5'- ATA TAC TCG AGG GGG GGC CCG GTA CCC AGC -3'
M: 5'- TAG CGC TCG AGG TCG ACG GTA TCG ATA AGC TTG AT -3'
T7: 5'- AAT ACG ACT CAC TAT AGG CGA ATT GG -3'

Using the T7 (forward) and M (reverse) primers, the amplicon containing the oriP ΔDS region was 2100 bp long. The PCR reaction consisted of 25 cycles of denaturation (60 s at 95°C), annealing (90 s at 62°C) and elongation (180 s at 72°C). Alternatively, using the G and M primer pair, the amplicon was derived from the whole plasmid (5.6 kbp) and the PCR conditions were: 31 cycles of denaturation (60 s at 95°C), annealing (90 s at 62°C) and elongation (420 s at 72°C). Given the large size of the amplicon, different conditions and PCR enhancing agents were tested. Optimized results were obtained with 10% (v/v) glycerol and Taq plus long (Stratagene, La Jolla, USA) specific for amplification of long templates.

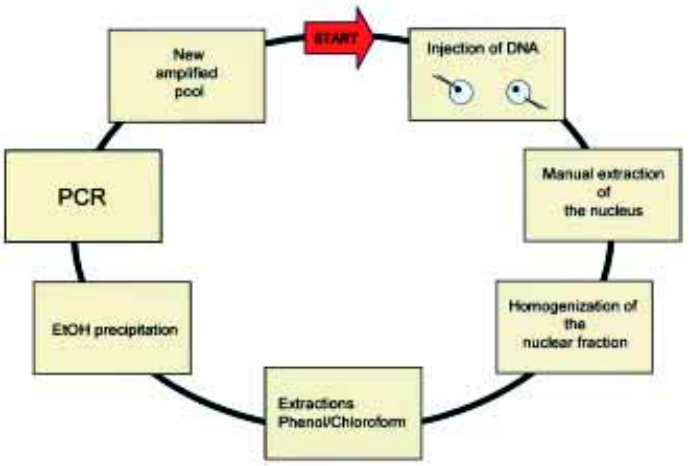

Fig. 2. Schematic representation of a combinatorial approach to select for DNA transport and nuclear retention elements. *For details, see the Experimental Procedures section. A pool* n *that starts (red arrow) the selection cycle ends up as pool* n+*1 (new amplified pool). A pool can in principle undergo many subsequent cycles of selection. For an explanation of the oocyte symbols, see legend to Fig. 1.*

The pool of oligomeric double-stranded DNA fragments was amplified by PCR using the PA and PB primer pair (Fig. 1B) which gives rise to an amplicon of 147 bp. PCR conditions consisted of 21 cycles of denaturation (60 s at 95°C), annealing (90 s at 55°C) and elongation (60 s at 72°C). Radioactive labelling of the DNA pool with [α-^{32}P] dCTP (Amersham; Braunschweig, Germany) was performed by PCR consisting of 30 cycles of denaturation (1 min at 95°C), annealing (90 s at 55°C) and elongation (60 s at 72°C). In addition, a 300 bp long fragment derived from a different plasmid (M. Pawlita, personal communication) was radioactively labelled by PCR under the same conditions, in order to obtain a larger fragment of double-stranded DNA for degradation studies. Degradation of this 300 bp DNA was also examined using the Bal 31 nuclease (MBI Fermentas; Vilnius, Lithuania).

PCR-amplified samples were loaded into agarose gels. The percentage of agarose within gels ranged from 0.8 to 2%, according to the size of the DNA fragments to be analysed. Agarose gels loaded with radioactively labelled DNA were dried with a gel dryer and a nylon membrane (Gene Screen Plus™, NEN, Boston, USA) was laid down in order to avoid the loss of the DNA fragments. Successive autoradiographs were obtained (Kodak X-OMAT Film) through exposition at –80°C with an intensifying screen.

Results and Discussion

Recovery of Plasmid DNA after Injection

Plasmid DNA microinjected into oocytes was detected by PCR amplification from cellular extracts. This PCR-based methodology is more sensitive than the direct isolation of radioactively labelled DNA from the extracts. We first examined the fate of the pTG 11220

plasmid (Fig. 1A), already known for its ability to be imported into the nucleus via sequences *in cis*. This plasmid contains the Epstein-Barr virus (EBV) oriP sequence. It has been shown that in mammalian cells expressing the EBNA1 EBV protein, pTG11220 exhibits increased nuclear import, accompanied by increased apparent expression of a plasmid-encoded gene (Längle-Rouault *et al*., 1998). Similar observations using viral systems have also been previously reported (Dean, 1997; Vacik *et al*., 1999). The 2100 bp long amplicon was found in the nucleus and cytoplasm when injected into the nucleus and cytoplasm, respectively. Interestingly, it was also detected in the nuclear fraction when the DNA had been injected into the cytoplasm (Fig. 1C, lane 1). This observation is compatible with findings in mammalian 293T cells (Längle-Rouault *et al*., 1998) and is indicative of the existence of nuclear import of plasmid DNA in *Xenopus* oocytes. Nevertheless, it remains uncertain if oriP sequences are functional in *Xenopus laevis*.

The amount of DNA generated by PCR varied. Maximal yield was found in the case of nuclear injection with recovery from the nuclear fraction (Fig. 1C; compare the 2100 bp band in lane 3 with that observed in lanes 1 and 2). We observed similar results upon amplification of the 5.6 kbp product (not shown). Both findings suggest that supercoiled DNA is highly stable in the *Xenopus* nucleus, which is consistent with results of previous studies (Wyllie *et al*., 1978). In order to test whether extracts can influence the PCR yield, different amounts of purified supercoiled DNA were amplified using the G, M primer pairs to generate the 5.6 kbp amplicon in the presence or absence of 10% oocyte extract (v/v). The presence of cellular extracts within the PCR reaction led to decreased recovery of amplified DNA (Fig. 1D). This effect is obvious when the intensities of the 5600 bp bands are compared. In particular, the band in lane 2 (1 pg of template DNA, no pre-incubation with oocyte extracts) is brighter than the band in lane 4 (10 pg of template DNA, pre-incubation with oocyte extracts)

Taken together, the results obtained with the pTG11220 plasmid suggest that DNA cis elements could be functional to DNA import into the oocyte nucleus, and that an inhibitory effect of oocyte components on PCR had to be taken into account.

These observations allowed us to extend our investigation about cis elements, and to this we considered a new kind of DNA probe, with a higher variability. This new probe consists of a pool of randomly generated linear DNA fragments, which can be produced *in vitro*. The main advantage of working with this pool is represented by the high degree of complexity of its random sequences, otherwise unreachable when injecting into the oocyte a single plasmid.

Selection of Oligomeric DNA Pools in Cell Extracts

This pool of random double-stranded DNA sequences (Fig. 1B) served to search for DNA elements mediating intranuclear transport or nuclear retention. Using PCR and specific primers, we detected DNA in both cytoplasm and nuclear extracts as well as in total oocyte extract following injection of the DNA pool into the cytoplasm or nucleus of oocytes, respectively (see Fig. 3; lanes 1 and 3). Furthermore, as in the case of plasmidic DNA, linear DNA was detected in the nuclear fraction subsequent to cytoplasmic microinjection (see Fig. 4), indicating that the linear DNA had been transported from the site of cytoplasmic injection to the nucleus.

In order to examine more systematically the putative cytoplasmic-nuclear transport of oligomeric linear double-stranded DNA, we carried out selection experiments. Considering that the central part of the DNA pool contains a 100 bp long randomised segment with a variability of 10^{17}, it is reasonable to assume that nuclear import may be facilitated by sequences in the randomised segment acting as functional cis elements. Furthermore, some sequences may also function as nuclear retention signals, a hypothesis which can in principle be tested by intranuclear injection and analysis of the remaining species. The selection was performed as schematically depicted in Fig. 2. Briefly, DNA (pool 0) injected into the cytoplasm or nucleus of oocytes was recovered from the compartment of interest, purified, amplified by PCR and the concentration was adjusted to 100 ng/μl (pool 1) before the next cycle was started by injection of the pool 1 into the cytoplasm or into the nucleus to isolate pool 2, and so on. In order to test the reproducibility of experiments, an assay was designed to check variability between two different animals (Fig. 3). Identical amounts of pools 0 and 1 were injected into 10 oocytes isolated from two different animals, and the DNA was isolated as described in Experimental Procedures. The amount of PCR products derived from the oocytes of two different animals was comparable if we take into account that *Xenopus* oocytes tend to show significant individual differences in physiological processes (Meissner *et al*., 1991).

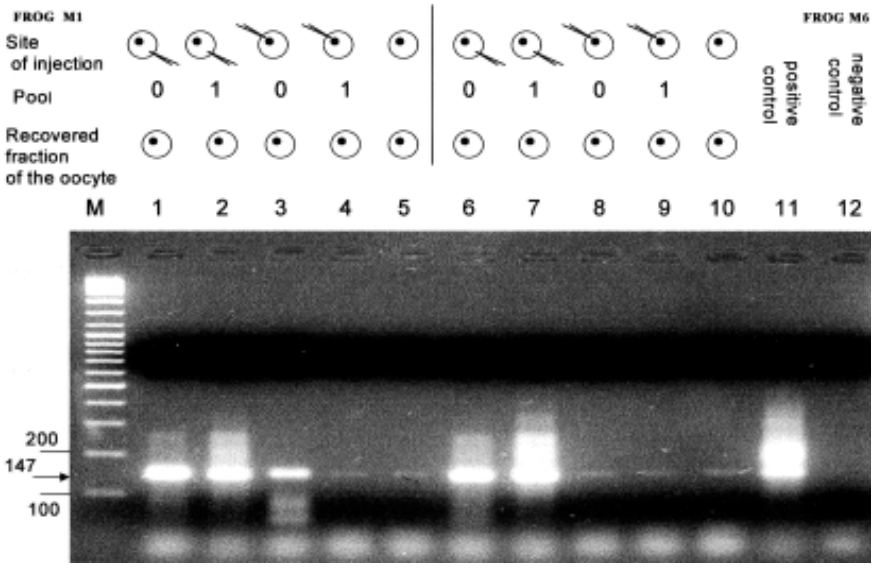

Fig. 3. Detection of cytoplasmic- or nuclear-injected DNA by PCR in oocyte extracts recovered from two different animals (M1 and M6) following injection of standard (pool 0) and one round-selected DNA (pool 1). *Equal amounts of pools 1 and 0 were injected into 10 oocytes from two different animals. Cellular extracts were prepared as described, used as template for amplification by PCR (21 cycles), and the products were separated by agarose gel electrophoresis. A comparison of the corresponding lanes (1 and 6, 2 and 7, 3 and 8, 4 and 9) indicates that the results obtained with two different frogs were reproducible. Lanes 5 and 10 refer to non-injected oocytes; a faint contaminating band is visible. Lane 11 represents the positive control with 1 ng of DNA as template and lane 12 is the non-template control. For an explanation of the oocyte symbols, see legend to Fig 1.*

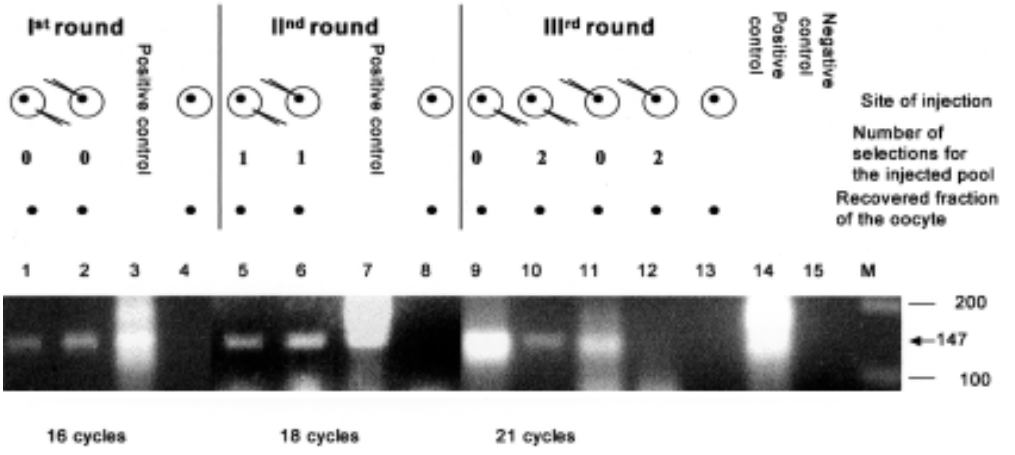

Fig. 4. Recovery of the DNA pool as a function of the number of selection cycles. *Each 147 bp band in the 2% agarose gel represents the PCR product of the pool recovered from a total extract of 10 oocytes. In the PCR positive controls (lanes 3, 7, 14), no cell material was present and the template consisted of 1 ng of linear DNA. The PCR negative control, in which no template was present, is shown in lane 15. The number of PCR cycles is indicated at the bottom of the corresponding panel. During selection cycles, the same amount of DNA was always injected (about 3x 10^{11}copies / oocyte), and every other experimental parameter was kept constant, so that eventual differences in band intensities after PCR are in principle due to the selection process itself. In particular, when one compares in the third round panel, lanes 9 and 11 with lanes 10 and 12, a substantial difference of band intensities between pools 0 and 2 can be appreciated. For an explanation of oocyte symbols, see legend to Fig. 1.*

The aim of the selection procedure was to isolate two different pools of fragments: those with an increased ability to migrate from the cytoplasm to the nucleus, and those that tend to be retained within the nucleus. The data relative to the cycle of selection of the random pool in oocytes are summarized in Table 1. Following 10 attempts, DNA could not be reproducibly detected by the fourth round of selection of cytoplasmically injected linear DNA. In the case of selection for nuclear retention, DNA could not be detected by the third round following a reasonable number (21) of PCR cycles. The number of the PCR cycles required to generate a measurable amount of product as evaluated by ethidium bromide staining increased with subsequent selection rounds. Thus, 16 cycles of PCR were needed to give a visible band for the first round of selection, 18 cycles for the second, and 21 for the third round. For example, during the third round of selection, pool 0 injected into the nucleus (positive control: Fig. 4, lane 11) gave rise to an amplicon after PCR amplification, whereas pool 2 injected into the nucleus and recovered under the same conditions (Fig. 4, lane 12) did not. Thus, this twice-selected pool apparently cannot be amplified using the standard protocol. Consequently, modified experimental conditions were tested in order to increase the yield of recovered low molecular weight DNA and allow further rounds of selection.

Modifications included: (i) sonication of the cellular extracts, (ii) treatment with up to tenfold increased concentrations of proteinase K, (iii) extending the time of proteinase K treatment from 1 hour to overnight, and (iv) increasing the temperature of proteinase K treatment to 56°C, at which the enzyme still conserves its highest activity (Sambrook *et al.*, 1989). To test whether chromosomal DNA or other impurities within the subcellular extracts affect the detection of the pool, DNA harvested after the selection cycle was diluted from 1:10 to 1:10^5 prior to PCR. However, this template dilution did not help to overcome the problem of low PCR yields. This result and the observation that the number of PCR cycles required to produce similar amounts of product was increasing during the selection process (Fig. 4, 16 cycles for the first step of selection, 18 for the second and 21 for the third), indicates that the random pool undergoes a negative selective pressure. Furthermore, two more extractions with pure phenol were performed, in addition to the ones indicated in the protocol and, to ensure a reproducible quality of the oocytes, in total 19 animals (M1-M19) were used for these experiments. Each animal was labelled and recorded to allow an appropriate time interval between the operations. In spite of all these measures, a significant improvement in the amount of recovered DNA was not achieved.

It could be argued that our evidence for cytoplasmic-nuclear transport could be due to the artifactual presence of perinuclear DNA in manually isolated oocyte nuclei, since it has been shown using confocal fluorescence microscopy that digitonin-permeabilised HeLa cells exhibit substantial amounts of perinuclear exogenous DNA even when no transport to the nucleus occurs (Hagstrom *et al.*, 1997). However, the fact that DNA was consistently not detected after the second round of selection, makes it unlikely that the DNA detected in the nuclear fraction is due to residual perinuclear DNA.

Integrity of Microinjected DNA

Since the loss of DNA during the selection process was likely due to the action of some nuclease, the fate of the radioactively labelled DNA pool was monitored after injection and subsequent incubation for 1 hour at 19°C. The isolation of low molecular weight DNA was performed according to the standard protocol. The samples were separated in 2% agarose gels and visualised by autoradiography. The results indicated that the cytoplasmic extract fraction consistently contained more linear DNA than the nuclear fraction, independent of the site of injection (data not shown). A similar experiment was performed in parallel *in vitro*, using a 300

TABLE 1

COPY NUMBER OF POOL SPECIES AFTER SELECTION ROUNDS

	First Round	Second Round	Third Round
Selection for Nuclear Retention	5X10^8	10^9	ND
Selection for Nuclear Import	5X10^8	10^9	10^7

Quantification of the amount of random pool molecules that went through each round of the selection process. The amount of molecules contained in the volume constantly injected in every round was 3x10^{11} molecules/oocyte. The values in the table refer to the number of molecules recovered by PCR from a single oocyte after injection. The fact that the total number of recovered molecules decreases during the selection process implies that, after each step of selection, a preparative PCR had to be performed, in order to inject always the same amount of molecules in the same volume. The quantification was obtained through a comparison of the intensities of the bands with samples containing known amounts of pool template DNA. ND, not detectable.

bp radioactively labelled DNA fragment which was incubated at room temperature overnight with separate nuclear or cytoplasmic fractions. This 300 bp fragment was preferred to the 147 bp random pool, because its bigger size facilitated the analysis of degradation processes. In order to monitor a possible nuclease activity, the separation of oocyte fractions for this experiment was performed without using TCA. Instead, enucleation was performed by holding the oocyte with a pair of watchmaker's forceps. Then a slit was pierced at the apex of the pigmented half with a syringe needle and, by gently squeezing the oocyte round its middle, the nucleus was extruded and transferred to a tube kept on ice (Hames and Higgins, 1984). The results obtained were similar to those obtained in the *in vivo* assays in that intact DNA could only be detected in samples incubated with cytoplasmic fractions (Fig. 5, lanes 4-6). No trace of the 300 bp fragment was visible after incubation with nuclear fractions (Fig. 5, lanes 1-3), even though a radioactive measure of the samples before being loaded into the gel gave approximately the same number of counts for every sample.

Highly stable cytoplasmic oligomeric DNA was also surprisingly observed in other experiments. Degradation was only observed when the 300 bp DNA fragment was incubated with cytoplasmic fractions to which 0.01 units of the Bal 31 nuclease and Mg^{2+} (10 mM) were added (Fig. 5, lane 6). These experiments *in vitro* are consistent with the previous assays with the DNA pool and both are indicative of the existence of a strong 5' → 3' exonuclease activity in the nucleus, which has been reported by another group (Maryon and Carroll, 1989). This potent nucleolytic degradation may explain why cycle-dependent enrichment of the DNA pool in the nucleus of *Xenopus* oocytes could not be observed in our assays. Moreover, the fate of exogenous DNA introduced into oocytes depends on its physical state and size. Circular DNA molecules, like the pTG 11220 plasmid used in this work, are relatively stable in the nucleus because they are inaccessible to the activity of the 5' → 3' exonuclease and over a period of a few hours circular DNA molecules can be assembled into chromatin (Gargiulo *et al.,* 1983; Carroll *et al.,* 1986). In contrast, linear molecules are not assembled into normal nucleosomes (Mertz, 1982). They can instead follow two further paths: they can be totally degraded exonucleolytically (Maryon and Carroll, 1989) or, if the fragments have homologous sequences near their ends, they can undergo homologous recombination (Grzesiuk and Carroll, 1987; Segal and Carroll, 1995; Lehman and Carroll, 1991), whereupon the ends can join together, inter- or intramolecularly. Nevertheless, it seems unlikely that the pool of random DNA fragments which we employed in this study serves as a substrate for endogenous recombination processes due to the oligomeric size of the DNA (147 bp) and its linear configuration. Even though there is evidence that DNA recombination is an effective process in the nucleus (Maryon and Carroll 1991), it has been shown that there are kinetic differences in the recombination process which depend on the length of DNA substrates. In the case of small DNA molecules, the 5' → 3' exonuclease degrades the substrate to completion before the slow step of recombination is accomplished (Carroll, 1996). Further evidence in support of degradation rather than recombination leading to the loss of pool DNA is provided by the finding that no high molecular weight recombination product was observed in the experiments with radioactively labelled DNA (data not shown). Furthermore, it should be borne in mind that PCR amplification could be prevented by degradation of even short parts of terminal sequences flanking the pool DNA and representing the primer binding sites.

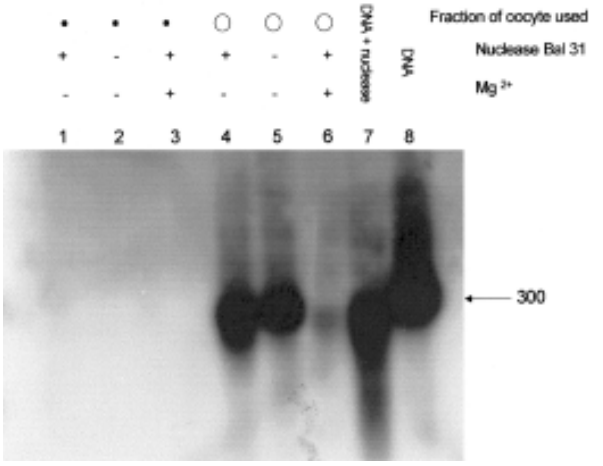

Fig. 5. Nuclease activity in subcellular fractions of *Xenopus* oocytes. *Autoradiography of radioactively labelled linear DNA after overnight incubation with nuclear or cytoplasmic oocyte extracts. Equal amounts (40,000 CPM per lane) of a 300 bp labelled fragment were incubated at room temperature overnight, either with nuclear (lane 2) or cytoplasmic (lane 5) extracts alone or with a supplement of Mg^{2+} (10 mM, lanes 3 and 6), and/or 0.01 units of Bal 31 nuclease (MBI Fermentas, Vilnius, Lithuania; lanes 1,3,4,6). After incubation, samples were directly loaded into a 2% agarose gel. Lane 7 contains DNA with 0.01 units of nuclease and lane 8 contains DNA only. No DNA was visible after overnight incubation with nuclear fractions (lanes 1-3). In contrast, DNA incubated with cytoplasmic fractions was partially degraded in the presence of exogenous nuclease (lane 4) and was intensely degraded when the nuclease was accompanied with an excess of Mg^{2+} (lane 6). For an explanation of the oocyte symbols, see legend to Fig. 1.*

It is apparent from the data in Table 1 that the selection for nuclear import and retention is not symmetrical (see ND versus 10^7 in column "Third Round"). This asymmetry may be due to the dependence of the efficiency of the nuclease on the path followed by the DNA to reach the nucleus. If the DNA is directly injected into the nucleus, it may be readily attacked by the nuclease and degraded. If the DNA is first injected into the cytoplasm and later transported into the nucleus, it may bind cytoplasmic proteins which could slow down the action of the nuclease.

The asymmetry which we consistently observed in the selection for nuclear import versus nuclear retention, could reflect not only the different DNA-interacting protein compositions in cytoplasm and nucleus, but also the existence of a difference in the composition of the selected pool. One could hypothesise that nuclear injected DNA pool consists of two groups of molecules. The first group would not contain any DNA cis element facilitating nuclear retention and would be small enough to freely diffuse throughout the nucleus, eventually reaching the cytoplasm through the nuclear pores without any energy or signal requirement (Zanta *et al.*, 1999). The principal factors hindering this free diffusion of DNA towards the cytoplasm include the restricted intranuclear motion of the DNA, its tendency to bind to different nuclear DNA binding proteins (Misteli, 2001), and a rather low nuclear pore density in the nuclear membrane (Maul and Deaven, 1977). The second group of pool species would be composed of molecules that tend to associate with nuclear proteins, thereby being compelled to stay inside the

nucleus. Here they undergo faster degradation than molecules which were first injected into the cytoplasm, then complexed to cytoplasmic proteins and finally imported into the nucleus. This may be due to differences in DNA accessibility concerning nuclease activity localised in the nucleus.

Conclusion

Our data indicate that linear double-stranded DNA is surprisingly stable inside the *Xenopus laevis* oocyte cytoplasm. Our results support the idea that this DNA enters the nucleus and induces increased nuclease activity as the total amount of exogenous DNA increases inside the nucleus, leading to stronger degradation of DNA. The presence of this strong 5'→ 3' DNA exonuclease activity in the nucleus does not necessarily invalidate the efficacy of our combinatorial approach since appropriate modifications to the extremities of the DNA fragments to protect them from the nuclease can be performed. For instance, the addition of defined DNA stretches on both sides of the fragments could be carried out, reducing the probability of exonuclease degradation in the primer binding region. In this way, the technique which we report has the potential to become a powerful biological tool to rapidly and efficiently identify new cis elements which function, for example, as NLSs. This new technique offers two main advantages. The first one is the possibility, thanks to the *in vitro* oligonucleotide synthesis, of screening a large number of DNA sequences containing putative cis elements. In addition to it, the sensitivity of the PCR amplification allows the isolation of even rare DNA sequences that survived the exonuclease degradation in the oocyte nucleus. Creation of constructs consisting of these new NLSs and the desired transgene could lead to increased nuclear uptake of DNA during early embryo development and to enhanced frequency of transgene integration into the germline of founders, as already observed in similar studies performed using the zebrafish egg system (Collas and Alestrom, 1996, 1997a,b, 1998).

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