Association of DNA with nuclear matrix in in vitro assembled nuclei induced by rDNA from *Tetrahymena shanghaiensis* in *Xenopus* egg extracts

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Abstract Nuclei assembled in vitro from *Xenopus* egg extracts and DNA display many properties known from in vivo nuclei. However, the distribution pattern of DNA in such nuclei remains unknown. We introduced rDNA from *Tetrahymena* macronuclei into *Xenopus* egg extracts and examined the association of specific DNA sequences with the nuclear matrix(NM) of the nuclei formed. We found that the 5′ non-transcribed spacers (5′-NTS) specifically bound to the NM as previously shown in normal *Tetrahymena* macronuclei.

Key words: Nuclear assembly; Nuclear matrix; *Xenopus* egg extract; *Tetrahymena* rDNA

1. Introduction

In 1959, Sze et al. [1] microinjected exogenous DNA into amphibian eggs and found that the DNA formed nuclei-like structures surrounded by a nuclear envelope. In the 1980s Newport et al. [2] showed that nuclear-like structures assembled around exogenous protein-free DNA incubated with *Xenopus* egg extracts. Such nuclei display many functions of normal eukaryotic nuclei, such as DNA replication and protein transportation through nuclear pore complexes. Later the formation of nucleosomes and typical chromatin structures was also demonstrated in such artificial nuclei [3,4]. However, distribution patterns of DNA in such nuclei remains unknown.

In 1974 Berezney and Coffey [5] demonstrated that after removal of DNA and high-salt extraction of chromosomal proteins, some residual proteins remain which constitute a 'nuclear skeleton' to which chromosomal DNA appears to be attached. Later the term 'nuclear matrix' (NM) was introduced for this structure and the idea that this NM is involved in the organization of the eukaryotic genome has steadily gained support. Cai and Zhai [6] demonstrated that such a NM also exists in the in vitro assembled nuclei, and this raises the question whether this NM also controls the distribution of DNA in these nuclei.

The ribosomal DNA (rDNA) of *Tetrahymena shanghaiensis* is a good model to study the distribution of a specific DNA in nuclei. The association in vivo of ribosomal genes with the NM has been reported in several studies (e.g. [7]). Furthermore it was shown that the 5′ non-transcribed spacers (5′-NTS) flanking the coding region and the telomere of rDNA in *T. shanghaiensis* serve as anchorage sites for the whole ribosomal gene in the macronuclei [8].

Here we report that rDNA could induce nuclear assembly in *Xenopus* egg extracts. When we compared the association affinity of different regions of rDNA with the nuclear matrix in these nuclei, we found that the regions binding to the NM were localized in the 5′-NTS flanking the transcribed region, as in *Tetrahymena* macronuclei.

2. Materials and methods

The rDNA used to induce artificial nuclei was isolated from *Tetrahymena shanghaiensis so. nor.* cultures, which were provided by Prof. Yeuzeng Chen (College of Life Sciences, Peking University). The cells were cultured in 2% protease peptone (Oxoid), 0.5% yeast extracts (Oxoid), 0.1% glucose, with gentle shaking (150 rpm) in a water bath adjusted to 27°C. Later (24–48 h), the rDNA was isolated and purified according to the method of T. Cech [9].

To explore possible anchorage sites for the *rRNA* gene to the NM of artificial nuclei, we used the plasmid pRd4-1, a gift from Prof. E. Blackburn (University of California, San Francisco). In this plasmid the rDNA is inserted in pBR322 (Fig. 1). After digestion with endonuclease *Hin*dIII and electrophoresis, the *rDNA* gene (fragments of 4.2, 2.2 and 1.6 kb) was recovered and labelled with 11-biotin-dATP or [^{32}P]dCTP by random primer labelling and used as probes for in situ hybridization and dot hybridization.

Xenopus egg extracts were prepared by the method of Zhang et al. [3]. *Xenopus* eggs were dejellied with 2% cysteine (pH 7.8), rinsed twice in MMR (0.1 M NaCl, 2.0 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.0 mM HEPES, and 0.1 mM EDTA, pH 7.8), activated by 0.2 μg/ml Ca^{2+} ionophore A23187 (Sigma) in MMR, then lysed by centrifuging twice at 10 000×*g* for 15 min. The supernatant, representing the cytosol, was recovered to be used for in vitro nuclear assembly. rDNA (8 μg) was mixed with 200 μl of cytosol and an ATP-regenerating system (2 mM ATP, 20 mM phosphocreatine and 50 μg/ml creatin kinase) and incubated at 22°C. After 3–4 h, in vitro assembled nuclei could be observed.

For ultrathin section electron microscopy, the samples were fixed in 2.5% glutaraldehyde, post-fixed with 2% OsO_4 in 0.1 M PBS (pH 7.4) and embedded in Epon 812.

For electron microscopy in situ hybridization, samples were fixed in freshly prepared 3% paraformaldehyde in 0.1 M sodium cacodylate

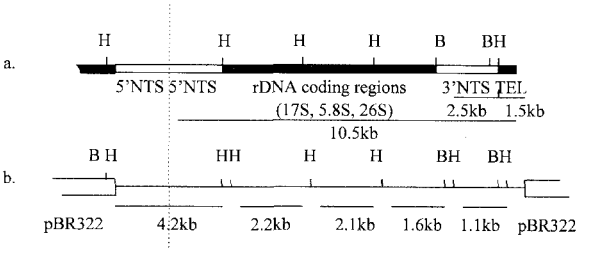

Fig. 1. Structure of palindromic rDNA (a) and pRd4-1 plasmid (b). Vertical dashed line indicates the center of the palindrome. A non-transcription region (5′-NTS) is located close to the center of the molecules. Downstream to the coding regions are other NTS (3′-NTS). At both ends of the DNA, there are telomere sequences (TEL). B, *Bam*HI; H, *Hin*dIII.

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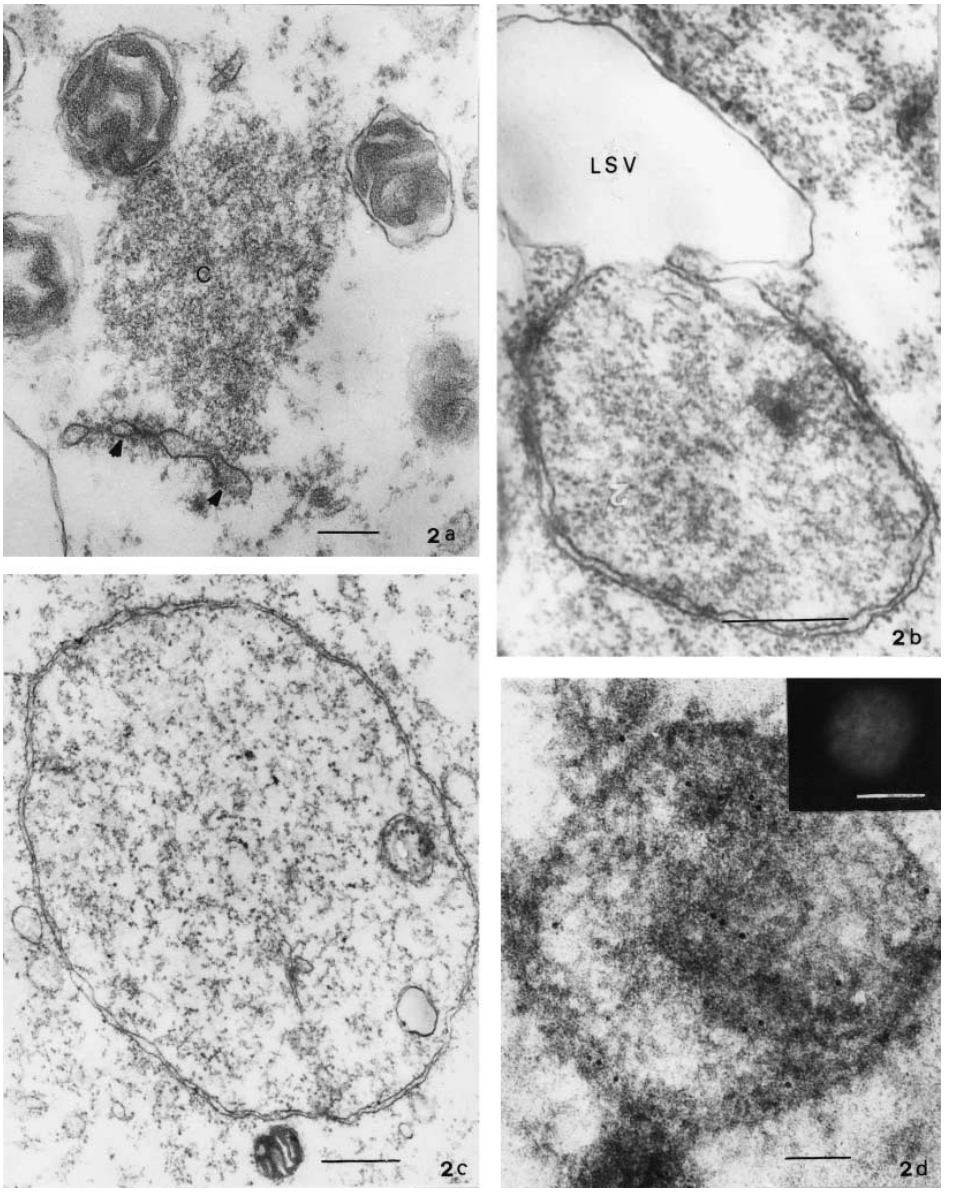

Fig. 2. Electron micrographs of ultrathin sections illustrate the nuclear-like structures formed by rDNA in *Xenopus* egg extracts. a: Early stage in nuclear assembly: chromatin-like structure (C) was formed from the rDNA. At the arrowheads membraneous vesicles are seen, attached to the periphery of the chromatin, the beginning of nuclear envelope assembly. Bar = 0.2 μm. b: A large smooth membrane vesicle becomes inserted into the nuclear envelope. Bar = 0.5 μm. c: Electron micrograph of an assembled nucleus, showing a typical double-membraned nuclear envelope and chromatin-like structures. Bar = 0.5 μm. d: Section of a Lowicryl K_4M embedded nucleus, hybridized with biotinylated pRd4-1 probe containing rDNA and detected by anti-biotin and gold-labelled (10 nm) secondary antibodies. Bar = 0.1 μm. Inset: After 4 h incubation at 22°C, the specimen was fixed and stained by DNA-specific dye (DAPI). Most of the DNA is now incorporated into nuclear-like structures. Bar = 5 μm.

buffer (pH 7.4), containing 0.1% glutaraldehyde and 4% sucrose, for 4 h at 4°C. After four washes, each for 15 min at 4°C in 0.1 M sodium cacodylate buffer containing 4% sucrose, the samples were embedded in Lowicryl K_4M.

Electron microscopy in situ hybridization was carried out using the method of Jiao et al. [10]. rDNA (fragments of 4.2, 2.2 and 1.6 kb) was recovered and labelled with 11-biotin-dATP as probe. For control, identical treatment with identical reagents was provided except that non-biotinylated pRd4-1 DNA was used instead of a biotinylated probe.

All above grids were stained with 2% aqueous uranyl acetate and lead citrate, then examined with a JEM-100CX.

Nuclear matrix was prepared according to Cai et al. with some modification [6]. The nuclei were extracted in cytoskeleton buffer (CSK: 100 mM KCl, 300 mM sucrose, 100 mM PIPES, pH 6.8, 3 mM $MgCl_2$, 1.2 mM PMSF, 0.5% Triton X-100) for 5 min. Soluble proteins were removed by centrifugation at 600×*g* for 5 min, and the sediment was extracted in RSB-Magik (42.5 mM Tris-HCl, pH 8.3, 8.5 mM NaCl, 2.6 mM $MgCl_2$, 1.2 mM PMSF, 1% (v/v) Tween 40, 0.5% sodium desoxycholate) for 5 min and centrifuged as before. The pellet was resuspended in digestion buffer (the same as CSK buffer except with 50 mM NaCl instead of KCl). Then ammonium sulfate was added to a final concentration of 0.25 M and incubated for 5 min. After centrifugation at 1000×*g* for 5 min, the pellets were resuspended in digestion buffer, and digested with 50 μg/ml DNase I or 20 μg/ml *Hin*dIII at 37°C for 4 h. Nuclear matrix and supernatant were obtained by centrifugation at 4000×*g* for 5 min after digestion. The sediments (nuclear matrix) were resuspended in proteinase K buffer (10 mM Tris-HCl, pH 7.8, 0.05 M EDTA, 0.5% SDS), and digested with proteinase K (50 μg/ml) at 55°C for 5 h. The super-

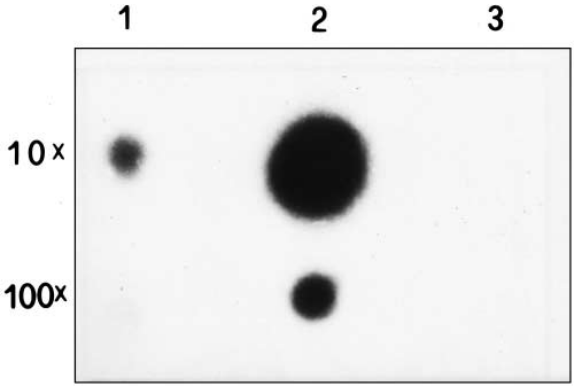

Fig. 3. Autoradiography of DNAs after they dot hybridized with [^{32}P]dCTP labelled pRd4-1 probe. Lane 1: rDNA as positive control; Lane 2: residual DNA bound to the nuclear matrix (NM). Lane 3: λ phage DNA as negative control.

natant DNA (SN DNA) and nuclear matrix-associated DNA (NM DNA) were obtained by extraction with phenol/chloroform and precipitation in alcohol.

For dot hybridization, we denatured the DNA samples at 95°C for 5 min followed by addition of 4 volumes of 20×SSC (SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0), and dotted them onto nitrocellulose (NC) filter according to the standard method.

For Southern analysis of the plasmid DNA, pRd4-1 was digested by *Hin*dIII, electrophoresis was performed in 0.7% agarose gel and transferred onto the NC filter. The supernatant DNA and nuclear matrix-associated DNA were labelled with [^{32}P]dCTP by random primer labelling and used as probe.

Hybridization was performed by the standard protocol.

3. Results

3.1. Nuclear assembly with purified rDNA and analysis of DNA components in the assembled nuclei

After incubation with *Xenopus* egg extracts, rDNA induced the formation of nuclear-like structures (Fig. 2c). Nuclear reconstitution occurred in steps and the ultrastructure of the assembled nuclei was similar to that induced by other exogenous DNA or chromatin previously reported (Fig. 2a,b) [2].

DAPI staining showed that after 3 h incubation most rDNA had assembled into nuclei (Fig. 2d, inset). We further visualized the rDNA in the nuclei at high resolution using non-isotopic electron microscopy in situ hybridization. After hybridization the biotinylated hybrids were detected by anti-biotin antibodies and secondary antibodies conjugated with 10 nm colloidal gold. The gold particles could be observed mainly distributed inside nuclear-like structures as shown in Fig. 2d. That indicated that the assembled nuclei contained rDNA molecules.

3.2. rDNA is associated with the nuclear matrix of in vitro assembled nuclei

Wang et al. [11] showed that even after excessive digestion with DNase I (50 μg/ml) for 6 h, 9% of total DNA was left bound to nuclear matrix. They proposed that the DNA contained some specific regions that were closely associated with the nuclear matrix. As we mentioned before, Cai proved that there is a nuclear matrix filament network in the nuclei assembled in *Xenopus* egg extracts. We therefore asked whether the exogenous DNA used had specific binding affinity for the nuclear matrix produced in a *Xenopus* cell-free system. Therefore the NM system was isolated from nuclei assembled from rDNA, then excessively digested by DNase I to obtain residual DNA. Dot hybridization with pRd4-1 containing rDNA showed that the residual DNA in the NM consisted of rDNA (Fig. 3).

3.3. Comparison of NM binding affinity of different regions of the rDNA

Is the association of rDNA with nuclear matrix random or specific? The NM system was isolated and digested with *Hin*dIII for 3–4 h at 37°C to obtain nuclear matrix-associated DNA (NM DNA) and supernatant DNA (SN DNA). Equal amounts of both DNAs were labelled by [^{32}P]dCTP and used as probes in Southern blotting with fragments of pRd4-1 digested by *Hin*dIII, respectively (Fig. 4). Fig. 4 showed the fragments containing 5′-NTS reacted with NM DNA positively, while the fragments containing the coding region and 3′-NTS were rich in SN DNA. These results suggest that the 5′-NTS is closely associated with nuclear matrix, while the transcriptional region and 3′-NTS have no relation to the nuclear matrix.

4. Discussion

In the 5′-NTS of each *T. shanghaiensis* rDNA molecule, there are three kinds of conserved repetitive sequences named types I–III, respectively [12]. Many previous studies have shown that these repetitive sequences are components of replication origins or transcription promoters and involved in the initiation of transcription or control of replication [13,14]. Further analysis of these sequences showed that they are highly AT-rich and DNase I-sensitive sites. These properties of the 5′-NTS regions suggest that they may bind to the nuclear matrix and thus play important roles in chromatin organization and control of gene replication and transcription. But very little is known about this DNA–protein interaction structure in the 5′-NTS. Do the proteins involved in transcription and replication interact with these regions in a transient manner or set up some stable DNA–protein interaction to help the organization of chromatin and nuclei, and

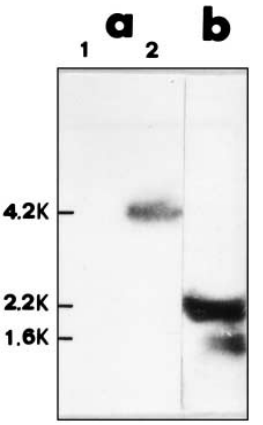

Fig. 4. The plasmid, pRd4-1, was digested by *Hin*dIII, run in 0.7% agarose gel, transferred to NC membrane, and hybridized with [^{32}P]dCTP-labelled NM DNA and SN DNA, respectively. a1: λ phage DNA *Hin*dIII fragments hybridized with NM DNA, as negative control. a2: pRd4-1 *Hin*dIII fragments hybridized with NM DNA. b: pRd4-1 *Hin*dIII fragments hybridized with SN DNA.

serve as recognition sites for transcription or replication factors?

Previous studies have shown that embryonic development in *Xenopus laevis* starts with a period of rapid cleavage without observable RNA transcription [15]. We found that nucleoli (indication of *rRNA* gene transcription) could not form even after active NOR (nucleoli organizer) were introduced into *Xenopus* egg extracts because of rRNA transcription inhibition [16]. It also is known that no specific sequences are needed for inducing nuclear assembly in *Xenopus* egg extracts. The rate of replication in the in vitro assembled nuclei is high and yet no specific sequences, such as the ARS-containing sequences in yeast, are required for replication induction [17]. However, we have shown here that specific sequences are present in rDNA 5′-NTS which are responsible for binding the rDNA to some non-histone proteins of the NM. We propose that these rDNA–NM interactions help to organize the chromatin distribution in the nucleus and may serve as recognition sites for replication or transcription. The details of this interaction between specific DNA sequences and NM proteins are what we want to explore in our future work.

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