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FAST TRACKS

Determination of the Chromatin Domain Structure in Arrayed Repeat Regions: Organization of the Somatic 5S RNA Domain during Embryogenesis in *Xenopus laevis*

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Abstract The size of the DNA loop containing the *Xenopus laevis* somatic 5S RNA gene cluster has been estimated using a simple, precise and sensitive method that we have developed for use on any tandemly arrayed DNA repeat region, and was found to increase during development We have found that after the mid-blastula transition, when transcription is activated in the embryo, a subset of somatic 5S RNA genes becomes specifically associated with the nuclear matrix. This association correlates with the transcriptional activity of the 5S genes. J. Cell. Biochem. 102: 1140–1148, 2007.

Key words: Xenopus; 5S DNA; nuclear matrix; transcription

In eukaryotic nuclei and metaphase chromosomes chromatin is organised into loops or domains which range in size from 20 to 250 kb [Buongiorno-Nardelli et al., 1982; Jackson et al., 1990]. These loops are anchored to the nuclear skeleton or nuclear matrix, a structure visualised by extraction of nuclei with various agents including mild detergents and high salt (for review see Vassetzky et al. [2000b]).

DNA is anchored to the nuclear matrix via specific sequences, called SARs or MARs for Scaffold or Matrix-Associated Regions [Mirkovitch et al., 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986]. MARs are located within fragments ranging from 200 to 10,000 bp, and are generally A/T-rich. Some are found in non-transcribed regions, and in some cases, within introns. Other MARs are function-related and are found in the vicinity of enhancers, insulators, replication origins and transcribed genes [Vassetzky et al., 2000b].

Chromatin loop organization is an important epigenetic feature: a strong correlation is observed between chromatin loop size, replicons and units of transcription. This relationship is particularly clear in early development of *Xenopus laevis*, where both loop size and replicon size evolve simultaneously [Buongiorno-Nardelli et al., 1982; Jun et al., 2004]. Moreover, we have shown that there is also a transition from random to specific attachment of the chromatin loops to the nuclear matrix, and this attachment correlates with the specification of DNA replication origins [Hyrien et al., 1995; Vassetzky et al., 2000a].

In the present work, we have studied the organisation of the Xenopus gene domain containing the somatic 5S RNA gene. The haploid genome of *X. laevis* contains 400 copies of the somatic 5S RNA gene organised as tandem repeats in a single chromosomal location. The gene unit consists of the 120 bp somatic 5S RNA gene flanked at the 5′ end by 607 bp and at the 3′ end, by 161 bp [Peterson et al., 1980]. In *Xenopus* embryos during the first 12 cell cycles following fertilisation, transcription is totally repressed. After de-repression of transcription during the mid-blastula transition, the somatic 5S RNA gene is transcribed throughout development and in adult cells [Jerzmanowski and Cole, 1990].

We have developed a simple, precise and sensitive method for the estimation of DNA loop

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size in any tandemly arrayed DNA repeat region. Using this method, we have found that the average size of the DNA loop containing this domain varies during development. We have also found that after the mid-blastula transition, a transcriptionally active subset of somatic 5S RNA genes becomes specifically associated with the nuclear matrix.

MATERIALS AND METHODS

Nuclei

Nuclei from *Xenopus* embryos were isolated by the method of Gorski et al. [1986]. Nuclei were isolated from Xenopus liver as described by Marilley and Gassend-Bonnet [1989].

Nuclear Matrices

Nuclear matrices were prepared by treatment of the isolated nuclei with either NaCl or lithium 3,5-diiodosalicylate (LIS) essentially as described elsewhere [Gasser and Vassetzky, 1998].

NaCl Extraction

10^5 nuclei were extracted by addition of one volume of a buffer containing 4 M NaCl, 20 mM EDTA, and 40 mM Tris–HCl, pH 7.5 to obtain nuclear halos. The resulting nuclear halos were spun in a microfuge at 2,000*g* for 10 min at 4°C and then washed three times with a buffer containing 2 M NaCl, 10 mM EDTA, and 20 mM Tris–HCl, pH 7.5. Digestion buffer (100 mM NaCl, 25 mM KCl, 10 mM Tris–HCl, pH 7.5, 0.25 mM spermidine) was added to the halos to a final volume of 400 μl. The halos were digested with 1,000 u/ml *Hin*dIII for 3 h at 37°C. The resulting nuclear matrices were spun in a microfuge at 2,000*g* for 10 min at 4°C and then washed three times with a digestion buffer.

Nuclear matrices were digested with proteinase K and extracted with phenol–chloroform. The obtained nuclear matrix-associated DNA was treated with RNase A, and either was slot-blotted or radioactively labelled using the Ready-to-Go kit (Amersham) and used as a probe.

LIS Extraction

10^5 nuclei were extracted with five volumes of LIS extraction buffer containing 10 mM Tris–HCl, pH 7.5, 0.25 mM spermidine, 2 mM EDTA-KOH, pH 7.5, 0.1% Digitonin, and 25 mM LIS for 5 min at room temperature (RT).

The resulting histone-depleted nuclear halos were recovered by centrifugation at 2,500*g* for 20 min at RT. The pellet was washed three times in a washing buffer containing 20 mM Tris–HCl, pH 7.5, 0.25 mM spermidine, 0.05 mM spermine, 100 mM NaCl, and 0.1% Digitonin, and then digested with *Hin*dIII as above.

Nuclear matrices were digested with proteinase K and extracted with phenol-chloroform. The obtained nuclear matrix-associated DNA was treated with RNase A, and either was slot-blotted or radioactively labelled using the Ready-to-Go kit (Amersham) and used as a probe.

Loop Size Calculation

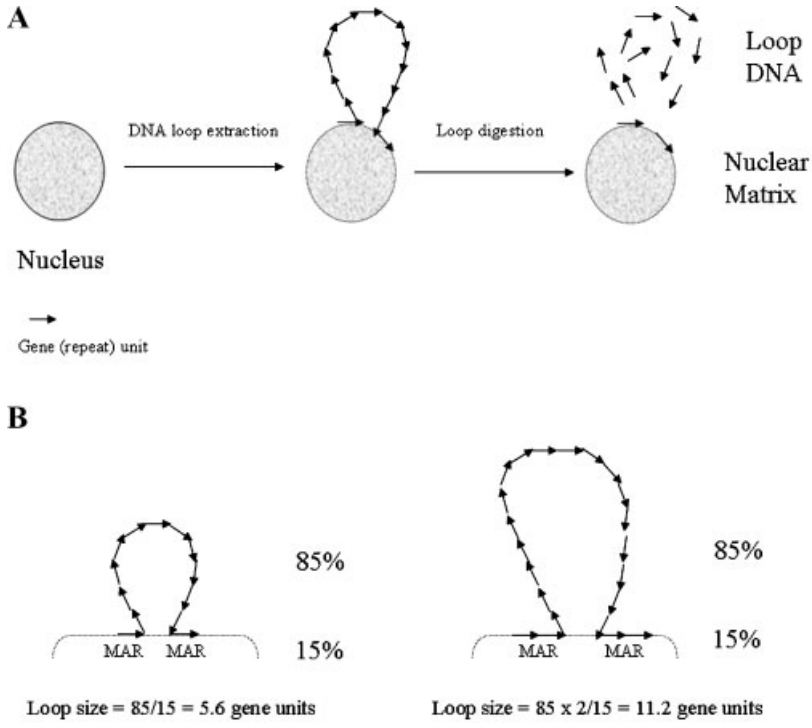
Loop DNA and DNA associated with the nuclear matrix were obtained by extraction of the isolated nuclei with either LIS or NaCl followed by treatment with *Hin*dIII. The loop (cleaved off) and the matrix (insoluble) DNA fractions were recovered and quantified by dot-blotting followed by hybridisation with a somatic 5S RNA gene-specific probe.

Knowing the size of the gene unit or the repeat unit, the size of the average attachment site and the percentage of units attached to the nuclear matrix (MARs), the average loop size in the locus can be calculated as follows: Loop size = ([%Loop DNA] × [average MAR size])/([%MARs] × [gene unit size]) (Fig. 1).

One half of the digested nuclei was used as the 100% control, the other part was extracted with NaCl or LIS and the nuclear matrix-associated fraction was separated from the loop DNA by several washes and centrifugation steps. Some material was lost during this process, therefore the loop and MAR fractions do not add up to 100%. We assume that a similar proportion of DNA was lost in both fractions, therefore we do not introduce any correction values in our calculations.

Calculation of the Average Numbers of Active Genes per Loop

The amount of structural nuclear matrix attachment can be estimated on the basis of the LIS extraction experiments. NaCl extraction experiment provides both structural and transcription-related MARs. Therefore the proportion of transcription-related MARs (TR MARs) can be calculated by subtraction: TR MARs = [%NaCl MARs] – [%LIS MARs]. The average numbers of active genes per loop can be



$$\text{Loop size} = \frac{(\%\ \text{loop DNA}) \times (\text{MAR size})}{(\%\ \text{MAR DNA}) \times (\text{gene unit size})}$$

Fig. 1. Principles of the chromatin loop size calculation. **A**: Nuclei are treated with either 2 M NaCl or 25 mM LIS and then digested with a restriction enzyme that cuts between the 5S rDNA gene units. The ratio between the gene content in the loops and the matrix is indicative of the loop size. **B**: The loop size calculations should be adjusted for different MAR sizes. Both panels show a situation where 15% of total DNA is found in the nuclear matrix. In the **left panel**, the MAR size is one gene unit, and in the **right panel**, two gene units. Note that the loops sizes are different in the two cases.

calculated as follows: [%TR MARs] × [Loop size]/[gene unit size].

Run-On Transcription

Run-on transcription was carried out essentially as described in Verheggen et al. [1998]. The isolated nuclei of the tailbud stage embryos were permeabilized in 20 mM Tris–HCl at pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.05% Triton-X 100, 5 μg/ml leupeptin, and 5 μg/ml pepstatin, washed in the same buffer without Triton X-100, and incubated for 20 min at 23°C in a buffer containing 100 mM KCl, 50 mM Tris–HCl at pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA, 25 μM S-adenosyl L-methionine, 0.5 mM of ATP, CTP, GTP, 5 μCi [^{32}P]UTP, 5 U/ml Rnasine, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. The incubation was followed by the nuclear matrix isolation by either LIS or NaCl extraction. Total, matrix-associated, and non-matrix RNA was purified and hybridised with the somatic 5S-specific probe immobilized on a Hybond N+ membrane.

RESULTS

Developmental Changes in Chromatin Loop Size in the Somatic 5S RNA Gene Domain

The genome is organised at several levels in the eukaryotic nucleus, from nucleosomes to the highest level of packaging in the interphase nucleus with loops or domains with sizes ranging from 20 to 200 kb which are attached to a proteinaceous structure called the nuclear matrix or scaffold. Many functions of the genome, e.g. replication, transcription and RNA

processing are associated with this nuclear matrix. These functions in turn lead to the transient association of transcribing and replicating DNA with the skeletal structures of the nucleus [Ciejek et al., 1983; Razin et al., 1986; Girard-Reydet et al., 2004; Jenke et al., 2004].

Several studies have reported an increase in chromatin loop size during development in *X. laevis*. In order to determine whether this increase correlates with the onset of transcription after the mid-blastula transition, we have measured the average loop size in the chromatin loop domain containing the somatic 5S RNA genes that are transcribed during embryogenesis.

We have developed a biochemical method for estimating chromatin loop size, in the regions of the genome containing tandemly repeated gene units, based on the extraction of chromatin loops with a mild detergent, lithium 3,5-diiodsalycylate (LIS), followed by digestion of the loops by restriction endonucleases that cleave between the gene units (Fig. 1A). The loop and matrix fractions are separated and the content of a specific gene (or repeat) is quantified by hybridisation. The ratio between the gene content in the loops and the matrix is indicative of the loop size:

Loop size (S) = % of the repeat unit in the Loop fraction (L) × repeat Unit size (U)/% of the repeat unit in the nuclear Matrix fraction (M) or

$$S = L \times \frac{U}{M} \quad (1)$$

While this formula gives a rough estimation of the loop size, it proves to be inaccurate in cases where the size of the nuclear matrix attachment site (MAR) exceeds that of the repeat unit (Fig. 1B).

In order to obtain more precise data, account has to be taken of the Average MAR Size (AMS) expressed in repeat units:

$$S = \frac{L \times AMS}{M \times U} \quad (2)$$

The average size of the MAR can be estimated by gel electrophoresis of the restriction enzyme-digested MAR fraction followed by hybridisation with a specific gene probe.

We have isolated the loop and matrix fractions from embryos at different stages of development and analysed the domain size by hybridisation and quantification with a somatic 5S RNA region-specific probe.

A pilot restriction endonuclease digestion was carried out to determine the time point when the amount of matrix-associated DNA did not decrease further (Fig. 2A). Depending on the source of nuclei, 1.5–4 h digestion was found to be sufficient.

The two major methods used for the isolation of the nuclear matrix; extraction of nuclei with 2 M NaCl or extraction with lithium-3,5,-diiodosalicylate (LIS), allow the recovery of different subsets of DNA sequences associated with the nuclear matrix. Extraction with LIS in low-salt buffers [Mirkovitch et al., 1984] gives a fraction which contains DNA fragments termed SARs for Scaffold Attached Regions or MARs for matrix-associated regions that are structural in nature [Roberge et al., 1988].

The second method by extraction of nuclei with 2 M NaCl followed by analysis of the matrix-associated DNA, revealed an enrichment in actively transcribed genes [Cook and Brazell, 1980; van der Velden et al., 1984; Stein et al., 1999] or replicating DNA [Cook and Brazell, 1980; van der Velden et al., 1984].

We have used these methods to measure the chromatin loop size and transcription-associated attachment of somatic 5S RNA genes with the nuclear matrix in pre- and post-MBT *Xenopus* embryos (stages 6 and 13) and also in adult somatic (liver) cells.

In order to accomplish this, we have extracted the isolated permeabilized nuclei, from either stage 6 or stage 13 embryos or from liver cells either with LIS or NaCl, and then separated loops from the nuclear matrix by *Hin*dIII digestion. The DNA fraction that was protected from digestion by the nuclear matrix proteins was retained within the nuclear matrix fraction while the unprotected loop DNA was cleaved off by the restriction enzyme. The matrix-associated and the cleaved-off DNA obtained by the two methods were extracted, re-digested with *Hin*dIII and analysed either by DNA electrophoresis in an agarose gel followed by blotting and hybridisation with a somatic 5S RNA region-specific probe or by slot-blotting followed by hybridisation (Fig. 2B). DNA sequences associated with the LIS-extracted nuclear matrices contain the points of anchorage of chromatin loops [Mirkovitch et al., 1984]. Knowing the size of the somatic 5S RNA unit, the size of the average attachment site (Fig. 2C) and the percentage of units attached to the

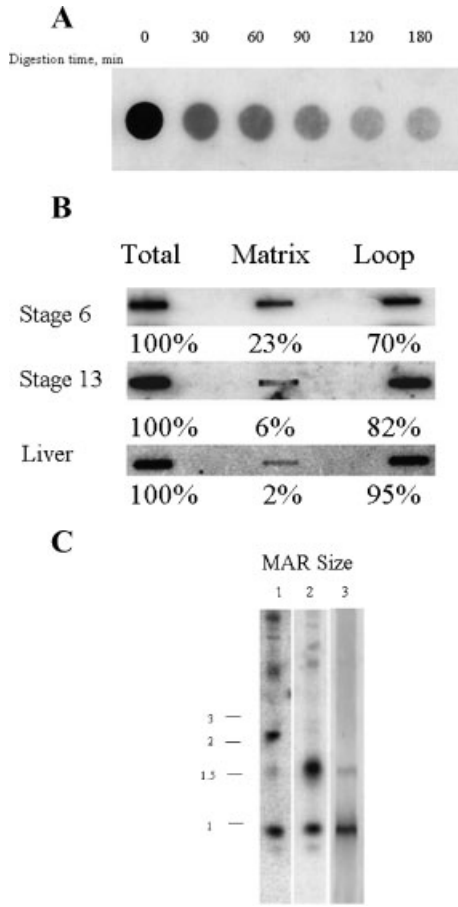


Fig. 2. Association of the somatic 5S RNA genes with the nuclear matrix in embryonic and somatic cells. **A**: Dynamics of the histone-depleted nuclei digestion by restriction endonucleases. Histone-depleted nuclei were obtained by treatment of the isolated nuclei from Xenopus adult liver cells with LIS. They were digested with *Hin*dIII at 37°C for different times. The DNA associated with the nuclear matrices was isolated as described in Materials and Methods and blotted. The blot was hybridised with the somatic 5S RNA gene probe. **B**: Quantitation of the nuclear matrix-associated somatic 5S RNA gene units in Xenopus embryos and tissues. Nuclear matrices prepared by treatment of the isolated nuclei from stage 6, stage 13 embryos or adult liver cells with LIS and digestion with *Hin*dIII. The DNA associated with the nuclear matrices as well as the non-associated DNA were separated, deproteinated as described in Materials and Methods and slot-blotted. The blot was hybridised with the somatic 5S RNA gene probe. All the experiments have been carried out in triplicate. Results of a single experiment are presented. Total, total nuclear DNA; Matrix, nuclear matrix-associated DNA; Loop, loop (cleaved-off) DNA. Stage 6, stage 6 embryos; Stage 13, stage 13 embryos, Liver, liver cells. **C**: Estimation of the size of the attachment site in the somatic 5S RNA locus. Nuclear matrices prepared by treatment of the isolated nuclei from stage 6, stage 13 embryos or adult liver cells with LIS and digestion with *Hin*dIII. The DNA associated with the nuclear matrix was extracted, run on an agarose gel, blotted, and hybridised with the somatic 5S probe. All the experiments have been carried out in triplicate. Results of a single experiment are presented. 1, matrix-associated DNA from the stage 6 embryos; 2, matrix-associated DNA from the stage 13 embryos; 3, matrix DNA from the liver cells.

nuclear matrix, we can calculate the average loop size in the locus.

In early embryogenesis the loops are quite small, 8.7 kb on the average, which is in perfect agreement with the replicon size in pre-MBT embryos. After the mid-blastula transition, we observe larger loops although they are still smaller than in somatic liver cells (21 kb vs. 61 kb, Table I).

Transcription-Related Association of Somatic 5S RNA Genes With the Nuclear Matrix

It is known that transcribing sequences constitute a part of the DNA associated with the nuclear matrices in salt-extracted nuclei (for review see Jackson [1997]). In stage 13 embryos, a significant portion of the somatic 5S RNA genes are associated with the nuclear matrix in the NaCl-extracted nuclei, while in the LIS-extracted nuclei this proportion is significantly lower (Table I). These data suggest that a large fraction of somatic 5S RNA genes in the post-MBT embryos are attached to the nuclear matrix via transcription complexes (see Discussion).

We have studied the association of the rDNA unit with the NaCl-extracted nuclear matrices by hybridisation of the nuclear matrix DNA from the DNase I-digested nuclei (Fig. 3). This method allows fine mapping of the MAR. Indeed, the rDNA gene unit showed preferential association with the nuclear matrix as compared to the intergenic spacer. These data are in perfect agreement with our previous observations [Vassetzky et al., 2000a].

This allows us to calculate the percentage of somatic 5S RNA genes that are active at any one time in the rDNA locus as follows: the amount of structural nuclear matrix attachment can be estimated on the basis of the LIS extraction experiments. NaCl extraction experiment provides both structural and transcription-related MARs. Therefore the proportion of transcription-related MARs (TR MARs) can be calculated by subtraction:

$$\mathrm{TRMARs} = [\%\mathrm{NaClMARs}] - [\%\mathrm{LISMARs}] \tag{3}$$

The average numbers of transcription units per loop can be calculated as follows:

$$\frac{[\%\mathrm{TRMARs}] \times [\mathrm{Loopsize}]}{[\mathrm{geneunitsize}]} \tag{4}$$

TABLE I. Loops and Transcription Units in the Xenopus Somatic 5S RNA Locus

	MARs (%)		Average MAR size (kb)		Average loop size (kb)	Percentage of transcribing genes	Average numbers of active genes per loop
Cell type	LIS	NaCl	LIS	NaCl	LIS	NaCl	
Stage 6 embryo	19.1 ± 5.0	21.0 ± 7.2	1.95 ± 0.2	1.75 ± 0.3	8.7	NA	NA
Stage 13 embryo	6.5 ± 2.1	23.1 ± 4.8	1.65 ± 0.4	2.45 ± 0.9	20.8	16.6	3.6
Adult liver	1.9 ± 0.4	5.5 ± 0.3	1.10 ± 0.1	2.51 ± 0.4	61.8	3.6	1.8

The percentage of MARs and the average size of a MARs within the 5S rDNA locus in Xenopus embryos and tissues have been determined as described in Materials and Methods using either LIS or NaCl extraction. The average size of DNA loops and the average numbers of active genes per loop were calculated as described elsewhere.

Our data suggest that approximately 20% of the genes are actively transcribed and thus associated with the nuclear matrix. This proportion is much less in non-proliferating liver cells (Table I) where the level of somatic 5S RNA gene transcription is lower.

In summary, 23% of somatic 5S RNA gene units are attached to the nuclear matrix in stage

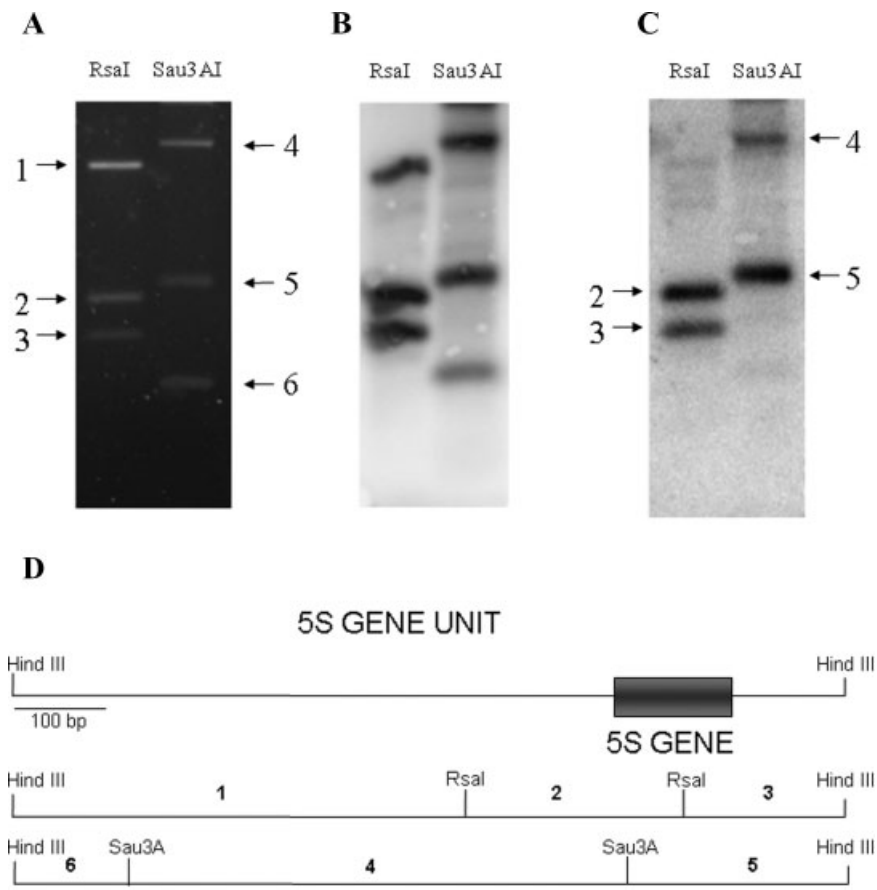


Fig. 3. Southern blot hybridisation of nuclear matrix-associated DNA from Xenopus embryos with the cloned fragments of 5S somatic RNA gene unit. **A**: Electrophoretic pattern of restriction fragments of recombinant DNA used for hybridisation with nuclear matrix DNA; (**B**) hybridisation with the total; (**C**) with the NaCl-extracted nuclear matrix-associated DNA from Xenopus embryos; and (**D**) the map of the somatic 5S RNA gene unit.

13 Xenopus embryos and approximately 80% of these attachments are transcription-associated.

Association of Transcriptional Complexes With LIS and NaCl-Extracted Nuclear Matrices

In order to assay the association of the transcriptionally active somatic 5S RNA gene copies in the nuclear matrices extracted with either LIS or NaCl, we have analysed the distribution of the nascent somatic 5S RNA transcripts in the nuclear matrix and the non-matrix fractions. Nuclei were isolated from stage 13 embryos and a run-on transcription was carried out in the presence of [α-32P]UTP as described in [Verheggen et al., 1998]. The nuclear matrix and the soluble fractions were isolated and the RNA extracted from both fractions was used as a probe to hybridise with the somatic 5S RNA gene. Figure 4 shows that $85 \pm 6\%$ of the nascent somatic 5S RNA transcripts were in the NaCl-extracted nuclear matrix fraction, indicating that the active somatic 5S RNA gene units were associated to with this fraction. Conversely, the LIS-extracted nuclear matrices contained only $15 \pm 3\%$ of the nascent somatic 5S RNA transcripts, confirming that most of the associations of the somatic 5S RNA genes with the nuclear matrix were structure-related in LIS-extracted nuclei.

DISCUSSION

The somatic 5S RNA locus in *X. laevis* is formed by tandemly arranged copies of the somatic 5S RNA unit comprising a 120 bp gene and a 760 bp spacer. Most, if not all somatic 5S RNA genes are located in a single locus within the distal end of chromosome 9 [Harper et al., 1983]. This unique structure allows us to estimate several features of the spatial organisation of the domain on the basis of analysis of its association with the nuclear matrix.

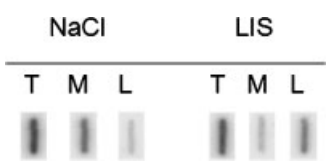


Fig. 4. Association of the nascent 5S RNA with the nuclear matrix in gastrula embryos. Run-on transcription in the presence of [^{32}P] UTP was carried out on isolated nuclei from tailbud stage embryos, followed by the nuclear matrix isolation using the LIS and NaCl extraction as described in Materials and Methods. Total (T), matrix-associated (M), and non-matrix RNA (L) was isolated and hybridised with the 5S gene-specific probe immobilized on a Hybond N+ membrane. The experiment has been carried out in duplicate. Results of a single experiment are presented.

DNA sequences associated with the LIS-extracted nuclear matrices contain the points of anchorage of chromatin loops [Mirkovitch et al., 1984]. Hence, knowing the size of the somatic 5S RNA unit, the size of the average attachment site (Fig. 2A) and the percentage of units attached to the nuclear matrix (Fig. 1), we can calculate the average loop size in the locus.

Early stages of Xenopus development proceed in an unusual manner: every cell cycle takes approximately 30 min, and replication initiates in individual chromosomes surrounded by nuclear membrane. These structures are named karyomeres [Lemaitre et al., 1998]. The average loop size in these structures is approximately 10 kb [Lemaitre et al., 1998], which is perfect agreement with our data on rDNA loop size during early development (8.7 kb). For structural, LIS extracted matrices in stage 13 embryo nuclei this gives a value of 20.8 kb. In embryonic cells, the loops are smaller than in somatic liver cells (20.8 kb vs. 60.8 kb, Table I). This is in agreement with the observation that an increased size of chromatin loop results in fewer structural attachment sites in differentiated as compared to embryonic cells [Buongiorno-Nardelli et al., 1982]. The chromatin loops coincide with the replication origins, and the replication cycle during early development is very rapid, and this requires small loop domains [Lemaitre et al., 2005 #1837]. Transcription of ribosomal genes starts after the mid-blastula transition, and it may cause torsional stress in small loop domains, therefore increased loop size in late embryos may represent a compromise between replication and transcription. In somatic cells, where there is no or little replication, the loops are big, which may be an advantage for transcription.

Interestingly, the average size of the attachment region is smaller in liver cells than in early embryos and seems to decrease during development. The digestion results are reproducible, therefore it is unlikely the longer MAR fragments in early embryos (Fig. 2C) are the results of incomplete digestion, but rather the observed decrease in the average MAR size is due to the nuclear organization and chromatin accessibility in early Xenopus embryos. Indeed,

we have earlier shown that the specificity of nuclear matrix attachment changes during development [Vassetzky et al., 2000a], therefore it is logical that the average MAR size may also vary.

Indeed, FISH analysis of chromatin loops of the 5S RNA gene domain comprising several hundred 5S RNA units in differentiated hamster cells indicate that the entire domain comprises only three loops, i.e. four sites of DNA attachment to the nuclear matrix [Nadel et al., 1995; Klaus et al., 2001]. Our recent FISH data confirm the conclusions of this biochemical assay [Petrov and Vassetzky, in preparation].

It is known that transcribing sequences constitute a major part of the DNA associated with the nuclear matrices in salt-extracted nuclei [Roberge et al., 1988; Jackson, 1997]. This allows us to calculate the percentage of somatic 5S RNA genes that are active at any one time in the locus. Our data suggest that approximately 20% of all the somatic 5S RNA genes are actively transcribed and associated with the nuclear matrix in post-MBT embryos. This proportion is significantly lower in non-proliferating liver cells (Table I) where the level of somatic 5S RNA gene transcription is lower.

Another characteristic that can be addressed on the basis of our data is the average number of active genes per loop. There are on average 3.6 units per loop in embryonic cells and half this number in the liver cells. One can argue that the presence of a higher number of actively transcribed genes per loop, which would associate with the nuclear matrix, could cause topological constraint on the DNA and the solution to this is to utilise more loops of a smaller size. In our calculation we have assumed here that actively transcribed units are dispersed evenly throughout the loops within the locus. We cannot however, rule out the possibility that all the active transcription units are on a small subset of loops. These possibilities could be resolved by mapping of the sites of active transcription onto the loops within the locus using FISH technique.

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