Transcription and methylation of flax rDNA

T.H.N. Ellis, P.B. Goldsbrough* and J.A. Castleton

John Innes Institute, Colney Lane, Norwich NR4 7UH, UK

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SUMMARY

The transcription of flax rDNA and the processing of the rRNA precursors is described. The location of methylated regions of the rDNA has been assayed, one of the least methylated regions maps close to the 5' end of the largest identified rRNA precursor.

INTRODUCTION

The organisation of the nuclear rDNA and 5S DNA of flax have been described [1,2]. The location of the 5S coding sequence has been inferred from DNA sequence data and it has been shown that the 5S DNA is extensively methylated in leaf tissue [2]. This paper describes the location of the rRNA precursor coding sequence on the rDNA map and this transcription map is compared to a map of the available HpaII sites. We conclude that the transcriptional strategy of the flax rDNA is akin to other rDNAs [3-7]. Structural parallels between the 5S DNA and rDNA can be clearly seen. Although these sequences vary independently of one another [8] they are subject to the same forces generating diversity and maintaining sequence homogeneity and gene fidelity.

MATERIALS AND METHODS

Nucleic Acid Manipulations

DNA was prepared from flax leaf tissue as described elsewhere [9]. RNA was prepared from flax callus cultures essentially by the standard Kirby procedure. The callus was derived from stem explants grown on Murashige and Skoog agar medium [10] supplemented with 5µM benzylaminopurine, 0.01µM naphthylacetic acid, and further cultured in the same liquid medium, but with 25µCi ml⁻¹ ³²P orthophosphate substituted for KH₂PO₄. The specific activity of this RNA was 15-40 x 10³ cpm µg⁻¹. Electrophoresis of glyoxalated RNA was according to the method of McMaster & Carmichael [11]. RNA was fractionated
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on 7-25% linear sucrose gradients in 0.15 M LiCl, 0.05 M Tris-HCl pH 8.0, 0.5% v/v SDS. These gradients were centrifuged at 23,000 rpm at 10°C for 16 hours in a Beckman SW41 rotor.

The methods used for agarose gel electrophoresis, transfer and hybridization have been described [8]. Transfers from 5% Acrylamide gels (19:1 Acrylamide : Bisacrylamide) were performed exactly as described by Southern [12] except that towels were left to blot 20 x SSC for two days.

Microdensitometry was performed using a Joyce-Loebl microdensitometer as previously described [13].

The enzymes HindIII, HpaI and II, MapI, PstI, SstI and SI were obtained from Bethesda Research Laboratories and the reaction conditions were as described by the supplier except that MapI was often used in vast (100x) excess. The enzymes EcoRI and BamHI were a gift of P. Dickinson, and standard assay conditions were used.

DNA fragment sizes were estimated according to the method of Southern [14] using the non-linear regression analysis of Duggleby [15].

Recombinant DNA techniques

i) Phage cloning

The phage collection in λ gt WES [16] was constructed by ligation of a partial EcoRI digest of flax DNA into λ gt WES DNA which had been self ligated, cut with SstI to destroy the internal EcoRI fragment, then phosphatased with calf intestinal phosphatase (Boehringer), phenol extracted, Ethanol precipitated and the redissolved DNA cut with EcoRI. This ligated DNA was then introduced to the E. coli strain ED8767 [17] by the process of in vitro packaging [18]. The frequency of wild type phage was estimated by packaging a control ligation in the absence of flax DNA.

All recombinant DNA techniques were performed in accordance with the directions of the Local Safety Committee.

ii) Construction of plasmids

The construction of the plasmid pBG 35 has been previously described [1].

The BamHI rDNA repeat of pBG35 was excised from its vector pAT153 [19] then recircularized and ligated. This circularized rDNA repeat was then re-inserted into the HindIII site of pAT153. This procedure separated the two HpaII-HindIII fragments of rDNA, and permitted their isolation as HpaII fragments of the reconstructed plasmid pRE1. These two HpaII fragments of pRE1 were isolated from 5% acrylamide gels and recloned into ClaI cut pAT153. (For a comparison of pRE1 and pBG35 HpaII fragments see...
The structure of the resulting Clal-HpaII clones pRH18 and pRH83 was verified in several ways:

i) Only one HpaII site from the rDNA was cloned as was shown by digestion of the plasmids with HpaII and comparison to pAT153 HpaII digests. (The mobility of the pAT153 HpaII fragment containing the Clai site was increased because insertion of a HpaII fragment into a Clai site destroys both the HpaII site and the Clai site).

ii) The plasmids hybridized to only one BamHI-HindIII fragment of rDNA in the plasmid pBG35. This locates which side of the HindIII site each plasmid represents.

iii) The hybridization pattern of pRH18 and pRH83 to HpaII and HpaII & HindIII digests of pBG35 fractionated on 5% acrylamide gels and transferred to nitrocellulose filters was as predicted.

The HpaII-HindIII fragment contained in pRH18 was also isolated directly by cloning HpaII-HindIII cut pBG35 into Clai & HindIII cut pAT153 and both this plasmid pJC28 and pRH18 behave identically when hybridized to flax DNA digests.

The BamHI-HpaII fragments were obtained by excising the HpaII fragment of rDNA in pRE1 which carries the BamHI site from the same 5% acrylamide gel as was used above. This HpaII fragment was ligated into the Clai site of pAT153 and plasmids containing this HpaII fragment in each orientation were obtained. From these two plasmids the small BamHI fragment was deleted by excision and recircularization. The structure of the two resulting plasmids pBJ1 and pBJ2 was verified by HpaII digestion, hybridization to BamHI and HindIII digests of pBG35 and hybridization to HpaII digests fractionated on 5% acrylamide gels prior to transfer to cellulose nitrate filters as before.

The HpaII-BamHI fragment of rDNA in pBJ1 was also obtained directly by excision of the corresponding HpaII fragment of pBG35 from a 5% acrylamide gel and cloning in the Clai site of pAT153. Again this plasmid pJC3 behaved like pBJ1 on blots of flax DNA.

SI nuclease mapping

SI nuclease mapping procedures were essentially as described by Berk & Sharp [20]. The hybridization temperature was determined by denaturing samples of BamHI digested pBG35 in 80% formamide buffer at 70°C and then incubating in 2°C increments at temperatures between 50°C and 62°C for 2 hours. Samples were then treated with SI nuclease and the DNA precipitated.
and analysed on agarose gels. No evidence of rDNA renaturation was observed at temperatures at or above 58°C. Approximately 700 ng of rDNA was used for hybridization reaction, RNA & DNA were mixed, the nucleic acids were precipitated with ethanol, and then redissolved in 10-20μl of 80% formamide, 0.4 M NaCl, 0.04 M PIPES pH 6.4, 1 mM EDTA. This mixture was heated to 70°C for 10 min to denature the RNA and incubated at 59°C for 2 hours. 20 volumes of ice cold 0.03 M Sodium Acetate pH 4.6, 0.05 M NaCl, 1 mM Zn acetate containing 100 units of SI nuclease (Bethesda Research Laboratories) was added and incubated at 27°C for 30 min. 5μg of tRNA and 3 vols of 90% ethanol were added and the nucleic acids precipitated overnight at -20°C. The precipitate was collected, dried and dissolved in 20μl of 0.03 M NaOH, 2 mM EDTA. Samples were electrophoresed under alkaline conditions [21] the gel neutralized in 3.0 M NaCl, 0.5 M Tris-HCl pH 6.5 and the DNA fragments transferred to cellulose nitrate.

Abbreviations Used

PIPES - Piperazine-N,N'bis[2 ethane sulphon acid]; 1,4 Piperazinediethane sulphon acid disodium salt (Sigma).

SDS - Sodium dodecyl sulphate (BDH specially pure grade)

SSC - 1x is 0.15 M NaCl, 0.01M trisodium citrate.

RESULTS AND DISCUSSION

Transcription of flax rDNA

i) Kinetics:

The most rapidly labelling abundant RNAs in flax callus cultures have been identified on Agarose gels. RNA was extracted from callus cultures incubated with 32P-orthophosphate (Amersham) for between 1/4 hour and 4 hours. The RNA was fractionated under denaturing conditions and the gel autoradiographed to reveal the sizes of labelled RNAs. Sizes of the RNAs have been estimated with reference to 32P labelled λ DNA digested with EcoRI and HindIII which had been glyoxalated and fractionated on the same gel.

From fig. 1 it can be seen that the first discrete RNA to be labelled is found in a band corresponding to a length of 7.0kb. This band was occasionally resolved as a doublet. The next two RNAs to accumulate label are found in bands corresponding to 2.6 and 4.0 kb. An RNA which is the sum of these two sizes would not have been resolved from the 7.0 kb band in this gel system. Label is eventually lost from these RNAs and is found to accumulate in RNAs whose length corresponds to the length of the mature rRNAs.

It seems likely that these rapidly labelling RNAs are precursors to
the mature rRNAs because the fate of the incorporated label is to be found in abundance in RNA molecules of the same size as the mature rRNAs. To provide more direct evidence for this, RNA preparations were examined to see if we could detect RNA molecules of the predicted sizes which would hybridize to rDNA sequences.

ii) rRNA precursors:

SI nuclease protection experiments [20] have been performed using the flax rDNA plasmid pBG35 [1] in order to define the location of the rRNA coding sequences in the rDNA repeat and to describe the structure of the rRNA precursor molecules. BamHI, HindIII or HpaI digests of pBG35 were hybridized with size fractionated callus RNA as described. Hybridization conditions were chosen which permitted the formation of RNA/DNA hybrids but in which DNA/DNA hybrids could not be detected. DNA fragments protected from SI nuclease by this hybridization procedure were detected after electrophoresis through agarose gels and transfer to cellulose nitrate filters, by hybridizing these filters with $^{32}$P-labelled [22] pBG35. Autoradiographs are
shown in figs. 2A, B, C.

The 18S and 25S RNAs are found in all the fractions and the 18S RNA is only found to be in excess in the lightest fraction assayed (F13). This is probably due to aggregation of the rRNA as previously discussed [27]. Because these fragments are detected in a uniform amount, it must be concluded that the hybridization reactions have been performed in RNA rather than DNA excess. No conclusion can therefore be reached about the relative abundance of the RNAs. However because of the sensitivity of the SI technique in detecting discontinuities in RNA-DNA hybrids the results on the size of the RNAs are not invalidated by the excess of RNA in the hybridization reactions.

From the patterns of protected fragments in these experiments it is relatively straightforward to deduce the positions of the ends of the RNA species with respect to the sites for BamHI, HindIII and HpaI in the rDNA. For example the 5.0 and 4.35 kb fragments protected in BamHI digests are replaced by fragments of 3.65 and 3.0 kb in HindIII digests. The difference between these two sets of sizes is due to the 1.35 kb distance between the BamHI and HindIII sites in the flax rDNA. (It should be noted that pBG35 is a BamHI monomer of flax rDNA repeat inserted in the BamHI site of pAT153 [1]; thus a discontinuity is always found at the BamHI site). It has thus been possible to define the location of the ends of the two RNA species on the rDNA map with respect to the sites for BamHI and HindIII. The size determination of these molecules is more reliably achieved by the SI technique than from the gels of glyoxalated RNA; these size estimates will be used subsequently. Clearly these techniques are in good agreement.

II) Processing:

The series of processing steps for the rRNA precursors has been defined. The 5′ end of the largest rRNA precursor has been mapped on the rDNA (fig. 3). The 3′ end of this large precursor defines the 3′ end of the 25S RNA. In addition four processing sites have been located. The sites 'a' and 'b' define the 5′ and 3′ end of the 18S rRNA respectively. The site 'd' defines the 5′ end of the 25S rRNA. The site 'c' lies between the 3′ end of the 18S rRNA and the 5′ end of the 25S rRNA. This may define the 5.8S rRNA.

The first processing event of the large precursor is a cleavage event at either the 'a' or 'b' sites; but these two sites both appear to be cleaved before either the 'c' or 'd' site is cleaved. This is deduced from the fact that the 4.05 kb RNA (25S RNA precursor) accumulates label before the mature 18S RNA, also the 2.5 kb RNA (18S RNA precursor) is not markedly
heterogeneous in length. The third and fourth cleavage events at sites 'c' and 'd' cannot be ordered, because cleavage of the 4.05 kb RNA (25S rRNA precursor) at the 'd' site prior to cleavage at the 'c' site would liberate mature 25S rRNA directly and the 'b'-'d' RNA may be either degraded or processed rapidly. It is not known whether cleavage at the 'c' or 'd' site releases 5.8S rRNA, which has been mapped to this location [1]. Although the 'b'-'c' fragment is nearer to the size of 5.8S rRNA than is the 'c'-'d' fragment. A prokaryotic sequence at the 5' end of the 23S rRNA is similar to the 5.8S rRNA [24] thus the 5.8S rRNA might be expected to derive from
the 'c'-'d' fragment.

This sequence of cleavage events (a or b) followed by (c or d) can be reconciled with information on the processing of RNP particles. If the RNA cleavage events which release mature 18S rRNA ('a' & 'b') are taken as an indication of the events which release the mature 40S ribosomal subunit from its 80S RNP precursor [25, 26] then it would seem that the cleavage at the 'c' or 'd' sites takes place on the 55S RNP particle after release of the 40S ribosomal subunit. Perhaps these sites are buried in the 80S RNP particle and only revealed after release of the 40S ribosomal subunit.
Fig 2. S1 nuclease mapping of rRNA precursors. RNA from the sucrose gradient fractions was hybridized with pBG35, and the fragments protected from S1 nuclease digestion were detected after fractionation on 1% alkaline agarose gels and transfer to nitrocellulose paper by hybridization with nick translated pBG35. λ DNA size standards were detected by the inclusion of nick translated lambda DNA in this hybridization reaction. Fraction 1 was towards the bottom of the gradient. T is total RNA. M is the lambda marker track. Relevant sizes are indicated in kb. The diagram shows the fractions in which the rRNA precursors are located, the sizes of the DNA fragments they protect, and the position of relevant sites in pBG35.

2A. BamHI cut pBG35 in protection hybridization.
2B. HindIII cut pBG35 in protection hybridization.
2C. HpaI cut pBG35 in protection hybridization.
Fig 3. Synthesis of flax rRNAs. The sequence of events leading to the synthesis of the mature rRNAs is indicated (top to bottom). The location of the large rRNA coding sequences on the rDNA is indicated along with the position of the 5' end of the largest transcript. The location of four processing sites a - d on this 6.55 kb RNA are indicated. The sequence of cleavage events at these four sites are indicated (top to bottom) and the sizes of the resultant precursor RNAs is given. The two alternative pathways are shown, but note that the 4.05 and 3.85 kb 25S rRNA precursors are common to both pathways.

In general terms this pattern of rRNA processing is very similar to that reported for other plants [3] and animals [4-7] except that in animals the pre 18S RNA appears to be an 18S RNA with a 3' extension rather than a 5' extension as in this case.

It should be noted that the largest rRNA precursor (6.55 kb) reported here is not proven to be the primary transcript from the rDNA. Neither has it been shown that the 5.9 kb rRNA is derived from the 6.55 kb molecule. However the simplest hypothesis is that there is only one initiation site for RNA polymerase I which transcribes an RNA of 6.55 kb which is subsequently processed. The existence of a pair of large RNA transcripts has been shown in other plants suggesting that either a double initiation event or this early processing at the 'a' site is common in plants. The
ratio of transcribed to non-transcribed rDNA in flax (ca 3) is high in comparison to other eukaryotes, which further lends support to the supposition that the 6.55 kb RNA is the primary transcript of the flax rDNA repeat.

Methylation of flax rDNA

i) CXG methylation:

The flax rDNA repeat carries a single site for the enzymes HindIII, BamHI and PstI. Of these three enzymes only HindIII reduces all the rDNA to the monomer size class in terminal digests. It is possible that these digests are partial either as a consequence of sequence heterogeneity or DNA methylation.

To distinguish between these two possibilities the digestion pattern of cloned rDNA repeats was examined. DNA was prepared from a lysate of a heterogeneous collection of phage (λgt WES [16]) which carried random EcoRI inserts of flax DNA. The collection was estimated to contain approximately 400 copies of each of the three EcoRI fragments of the flax rDNA. It can be seen from fig. 4 that the 3.7 kb EcoRI fragment is completely cleaved by either BamHI or PstI digestion when this fragment is derived from the phage collection, but not from genomic DNA. Thus the partial digestion of genomic rDNA is not a consequence of sequence heterogeneity.

Microdensitometry of the EcoRI fragments of genomic DNA digests suggests that 0.50 ± 0.09 of the BamHI sites and 0.78 ± 0.08 of the PstI
Fig 5. Comparison of genomic and cloned rDNA.
A. H - HpaII digest, M - MapI digest, HM - double digest. 1% Agarose gel. Total DNA digests transferred and hybridized with nick translated pBG35. Sizes are indicated (kb).

B. Two rDNA clones pREI and pBG35 and their vector pAT153 digested with MapI (M) on MapI and HindIII (MH). 5% Acrylamide gel. Sizes are indicated (BP).

sites are blocked against cleavage in genomic DNA. Both these enzymes are blocked by methylation of cytosine residues in the symmetrical trinucleotide CXG [27, 28]. The PatI site (CTGCAG) contains two of these trinucleotides while only a quarter of all possible BamHI sites (GGATCC) contain the sequence. Correcting for this discrepancy the frequency of CXG methylation in the PatI site can be estimated as 0.53 ± 0.08 which is in good agreement with the frequency estimated from this BamHI site.

This level of CXG methylation is twice the level of CXG methylation
in the BamHI site of the flax 5S DNA [2] and approximately twice the overall frequency of C methylation in flax [30].

ii) CG methylation:

The frequency of CG methylation was investigated using the enzyme pair HpaII and MspI [29]. The pattern of HpaII and MspI digests of flax rDNA is complex with either of these enzymes reducing cloned rDNA (pBG35) to approximately 30 small fragments (fig. 5).

It is clear from this comparison that the HpaII/MspI sites are extensively methylated both at the CXG and CG sequence. (This CXG methylation has not been distinguished from sequence heterogeneity however the behaviour of the clone pBG35 upon digestion with HpaII or MspI suggests that base changes at this sequence are not common).

Because these digestion patterns are complex a mapping procedure using labelled subfragments of the rDNA monomer has been adopted. The idea behind this mapping procedure is essentially that of the Smith & Bernstein endlabelling method [31] except that a specific probe for ends of fragments is used in place of end labelling. The description of the construction of the probes is given in the methods section.

In order to map the position of the HpaII fragments using hybridization probes for the ends of fragments it is necessary to generate both a defined starting position for the map and to obtain probes which contain only DNA sequences between the defined start and the first HpaII site. Two starting positions have been chosen which are the HindIII and BamHI sites thus four end probes consisting of two HpaII-HindIII fragments and two BamHI-HpaII fragments are required.

The results of hybridization of the four plasmids pRH18, pRH83, pBJ1 and pBJ2, corresponding to the HindIII-HpaII and BamHI-HpaII fragments, to HindIII, HindIII and HpaII, HindIII and MspI or BamHI, BamHI and HpaII, and BamHI and MspI digested flax leaf DNA fractionated on agarose gels and transferred to cellulose nitrate filters is shown in figs 6 & 7. It can be seen that in the HpaII and HindIII digests a considerable amount of rDNA monomer remains undigested. (This cannot be deduced for BamHI-HpaII digests because some monomer fragments in this digest may be a result of recruitment of the HpaII monomers from higher BamHI oligomers). The same conclusion can be reached however from an examination of Ethidium Bromide fluorescence patterns of HindIII and HindIII-HpaII digests of flax DNA or such digests probed with any rDNA clone (data not shown). Microdensitometry of the rDNA monomer in HindIII and HindIII-HpaII digests of flax DNA (both from
Fig 6. Hybridization of pRH18 and pRH83 to genomic DNA digests. (M) - Marker track. (H) HindIII digest (HH) HindIII and HpaII double digest. (HM) HindIII and MspI double digest. pRH18 probe 1.5% Agarose gel, pRH83 probe 1% Agarose gel. The fragment arrowed in both pictures corresponds to cleavage at the HpaII site near the 5' end of the large transcript. In both cases the error per millimeter in size estimation at this position of the gel is ca 300 BP.

The hybridization patterns of pRH18 and pRH83 and of pBJ1 and pBJ2 (fig. 6 & 7) show that the HpaII sites are not randomly inactivated. On long exposures of these autoradiographs many bands can be seen, these bands presumably correspond to cleavage at each of the HpaII or MspI sites. However short exposures show only a small number of bands. These few bands correspond to cleavage at the most readily available HpaII sites. These available sites have been mapped with respect to both the BamHI and HindIII sites. (It has not been possible to determine the cleavage frequency at
Fig 7. Hybridization of pBJ1 and pBJ2 to genomic DNA digests. For each two exposures are shown. (M) Marker track. (B) Bam HI digest. (BH) BamHI and HpaII double digest. (BM) BamHI and MapI double digest. In both cases the fragment corresponding to cleavage at the HpaII sites near the 5' end of the large transcript is indicated. The error per millimeter in size estimation for these two positions is ca 350 BP for the 5.35 kb fragment (pBJ1 probe 1% agarose gel) and ca 220 BP for the 4.08 kb fragment (pBJ2 probe 1.5% agarose gel). In this experiment because some BamHI sites are uncut it is possible to generate fragments smaller than rDNA monomer which are entirely HpaII or MapI fragments. This is expected to confuse an analysis of these results which are merely to confirm the data obtained from pRH18 and pRH3; however in practice the contribution of such fragments to the patterns observed appears to be slight presumably because of the infrequency of cleavage at either the HpaII or MapI sites in BamHI methylated rDNA oligomers.

Each of these sites because of the cumulative nature of the frequency distribution involved [32] and because of non-uniformity of the transfer and hybridization procedures for different size classes). The bands arrowed in figs. 6 & 7 can be clearly seen to correspond to a single readily available HpaII site. With both the probes BJ1 and RH18 the space between this arrowed fragment and the rDNA monomer is seen to be devoid of
Fig 8. Summary map of the flax rDNA.
An rDNA dimer is illustrated for convenience. The location of the largest transcript and the 18S and 25S rRNA coding sequences are shown. The location of the sequences homologous to the four plasmids pBJ1, pBJ2, pRH18 and pRH83 is shown as is the direction in which each has been used to map available HpaII sites. The location of available HpaII sites identified with each of these plasmids is shown below the main diagram. Error bars in the location of these sites shows the error associated with a 1 mm difference in fragment mobility. These sites are only shown between the extreme HindIII (H) and BamHI (B) sites. The consensus position of available HpaII sites is indicated on the rDNA map (\(\nabla\)).

bands in the HpaII digests, However in the more extensive MapI digests fragments are found to hybridize in this region of the gel. It must therefore be concluded that the absence of bands from this region of the gel in HpaII and HindIII double digests is not simply a consequence of the cumulative cleavage frequency at all the HpaII sites which map within the arrowed fragments. When all the HpaII sites up to this one have been cut no more HpaII sites are available for cleavage. There are therefore two components in the rDNA; repeats which are fully methylated and those which are under methylated at some specific sites. The specific site corresponding to the arrowed fragments in fig. 6 & 7 is seen to map close to the 5' end of the large transcript of precursor rRNA fig 8.
CONCLUSION

The transcription and processing of flax rDNA is typical of both plant and animal rDNAs [3-7]. The redundancy of rRNA genes in flax seems typical of plants in general [33]. The pattern of methylation of the flax rDNA is similar to that in Xenopus laevis [34, 32] except that there are several available HpaII sites as well as that which maps near to the 5' end of the largest transcript. The flax rDNA also shows the CXG methylation pattern which is common in plants but not animals.

It is tempting to speculate that as with Xenopus the undermethylation of the rDNA near to the 5' end of the largest transcript is associated with gene activity [32]. Thus in the leaf tissue from which this flax DNA is derived 35% of the rDNA is completely methylated and by inference inactive. It would be expected that either meristematic tissue or the cell lineages leading to spore production would be the main sites of rRNA synthesis. Thus the redundancy of rRNA genes in this and many other plant species can be considered as analogous to the 5S RNA gene redundancy in amphibians.

The rDNA of flax shares some features with the functionally related 5S DNA. Both sets of sequence are highly redundant, variable in copy number, showing low sequence heterogeneity, and are heavily methylated [1, 2, 8, 13]. It seems reasonable to assume that the dynamic properties of both gene clusters would be similar, and that the same physical processes operate to generate copy number diversity in both sequences.

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*Present address: Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA

REFERENCES