Volume 241, number 1,2, 229–233 FEB 06577 December 1988

Identification of *Eco*RV fragments spanning the *Nα*-tubulin gene of *Physarum*

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Received 16 October 1988

The *Nα*-tubulin gene of *Physarum polycephalum* has an *Eco*RV site at codons 252/253. *Eco*RV digestion of *Physarum* DNA generated two *Eco*RV fragments per gene copy comprising both coding and flanking sequences. Hybridisation probes which included coding sequences upstream from the central *Eco*RV site cross-hybridised with another α-tubulin gene. Probes derived from either 5′- or 3′-flanking regions were gene-specific. These probes identified two *Eco*RV fragments in the haploid strain CLdAXE viz 5.4 kb (5′-fragment) and 6.2 kb (3′-fragment). The same two fragments were identified in *Eco*RV digests of DNA of the diploid strain M_3CVIII, and a second form of the gene was also identified comprising two fragments viz 5.0 kb (5′-end) and 5.5 kb (3′-end). Both forms gave rise to an identical 4.65 kb *Hin*dIII fragment as judged by restriction mapping.

α-Tubulin gene; *Eco*RV fragment; Plasmodium; *alt* B Locus; (*Physarum polycephalum*, Myxamoeba)

1. INTRODUCTION

Tubulin genes of *Physarum polycephalum* are periodically expressed during the naturally synchronous mitotic cycle of the plasmodial phase [1–3]. Their expression is also governed by a developmental switch during the transition from the myxamoebal to the plasmodial phase of the life-cycle [4,5].

We have cloned and sequenced the *Nα*-tubulin gene which is expressed in plasmodia but not in myxamoebae. It contains seven intervening sequences and is unusual in having a methionine codon at the 3′-end instead of the usual tyrosine codon [6].

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Four α-tubulin gene loci have been identified on the basis of the segregation of *Eco*RV restriction fragment length polymorphisms [7]. Three loci, altA, altC and altD contain one gene sequence, whereas the altB locus comprises at least two genes [7]. The cloned gene, *Nα*-tubulin, forms part of the altB locus [6].

Four *Eco*RV fragments define the altB locus of the haploid strain CLd [4,7], but the lack of gene-specific probes has hindered their assignment to particular genes. For example, the *Nα*-tubulin gene, which has an *Eco*RV site at codons 252–253 and thus spans two *Eco*RV fragments, was identified as part of the altB locus by using probes containing coding sequences [6] which we now show to be conserved between α-tubulin genes (see section 3). We have now used gene-specific probes derived from outside the coding region of the *Nα*-tubulin gene to identify its characteristic *Eco*RV fragments in the haploid strain CLdAXE and the diploid strain M_3CVIII. These data will aid us in studying the physical linkage of α-tubulin genes at the altB locus and in exploring the possibility that they are coordinately expressed during the mitotic cycle.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2. MATERIALS AND METHODS

The growth of amoebae and plasmodia and the extraction of DNA was as described in [6]. Restriction enzyme digests were carried out according to the manufacturers instructions. DNA fragments (4 μg per lane) were separated by agarose gel electrophoresis and transferred to nitrocellulose filters [8]. Probes (see fig.1a) were made by sub-cloning fractions of phage λ-Nα Tu [6] into bacteriophage M13 vectors or isolating double-stranded sub-fragments. M13 clones were labelled by the 'prime-cut' method [9], double-stranded probes were labelled by nick-translation [10]. Hybridisation conditions were 5 × SSC (SSC is 0.15 M NaCl/0.015 Na citrate, pH 7.0), 50% (v/v) formamide, 42°C for 18 h. Filters were finally washed in 0.1 × SSC/0.1% (w/v) SDS at 55°C for 1 h. Hybridised probe was detected by autoradiography using Kodak X-Omat film and intensifying screens at −70°C for 1–2 weeks.

CLdAXE and M_3CVIII DNA samples were always run on the same gel and subsequently hybridised as a set, to enable accurate 'side-by-side' comparisons of molecular masses to be made between fragments.

3. RESULTS AND DISCUSSION

The *N*α-tubulin gene has an *Eco*RV site at codons 252/253. Thus, two *Eco*RV fragments span the gene, one extending upstream and the other downstream from this site. The principal result of this work is that two fragments j (upstream sequences) and h (downstream sequences) span the *N*α-tubulin gene in the haploid strain CLdAXE. Fragments j and h and also fragments k (upstream sequences) and i (downstream sequences) were identified in the diploid strain M_3CVIII.

3.1. *Identification of* Nα*-tubulin gene-specific probes*

The location of the *N*α-tubulin gene, the central *Eco*RV site (at codons 252 and 253) and the organization of the eight exons and seven introns within the 4.65 kb *Hin*dIII fragment cloned into phage-λ NM1149 are all illustrated in fig.1a. The *N*α-tubulin gene was originally assigned to the altB locus by using two probes, namely a 1.65 kb *Hin*dIII/*Eco*RV fragment comprising 5′-flanking sequences and coding sequences upstream from the *Eco*RV site, and a 3.0 kb *Eco*RV/*Hin*dIII fragment comprising coding sequences downstream from the *Eco*RV site and 3′-flanking sequences [6]. The 1.65 kb 5′-end probe recognised fragments f, j and k and the 3.0 kb 3′-end probe identified fragments h and i [6]. However, sequences within the coding region of an α-tubulin multigene family tend to be highly conserved. This property was exploited in defining α-tubulin gene loci in *Physarum* [4]. The potential for cross-hybridisation is a disadvantage in studies of a single gene.

3.2. *The* Nα*-tubulin gene of strain CLdAXE comprising* Eco*RV fragments j and h*

The pairing of 5′- and 3′-*Eco*RV fragments of the *N*α-tubulin gene was resolved by using probes A and H to study the haploid genome of *Physarum* CLdAXE myxamoebae [11]. Probe A (5′-end) hybridised with fragment j (fig.1c) and probe H (3′-end) identified fragment h (fig.1d), in an *Eco*RV digest of haploid myxamoebal DNA. Thus fragments j and h correspond to one form of the *N*α-tubulin gene, which is found in haploid myxamoebae of strain CLdAXE.

3.3. *A second* Nα*-gene comprises* Eco*RV fragments k and i*

The observation that fragments j and h comprise one form of the *N*α-tubulin gene facilitates the interpretation of results previously obtained with the diploid strain M_3CVIII from which the *N*α-tubulin gene was isolated. The gene-specific probe A recognised fragments j and k but not f (see fig.1b). However, fragment f, in addition to fragments j and k, was recognised by the probes B, C and D derived from the coding region which are sub-fragments of the 1.65 kb *Hin*dIII/*Eco*RV fragment (not shown). Thus hybridisation of the 1.65 kb *Hin*dIII/EcoRV probe to fragment f is attributable to cross-hybridisation between different *N*α-tubuline gene sequences. Probe H identified the same fragments, h and i, as the 3.0 kb *Eco*RV/*Hin*dIII probe used previously [6]. The *Eco*RV fragments j, h, k and i of M_3CVIII can therefore be assigned unambiguously to the two forms of the *N*α-tubulin gene.

We have shown above that fragments j and h complete one allele of the *N*α-tubulin gene. We infer that fragments k and i, identified in the diploid strain M_3CVIII, span the other allele. In both forms the *N*α-tubulin gene is contained in a 4.65 kb *Hin*dIII fragment which is indistinguishable from the fragment which was cloned and sequenced, judged by the location of eight restriction sites within and around the transcription unit (see fig.2). We infer that the nucleotide

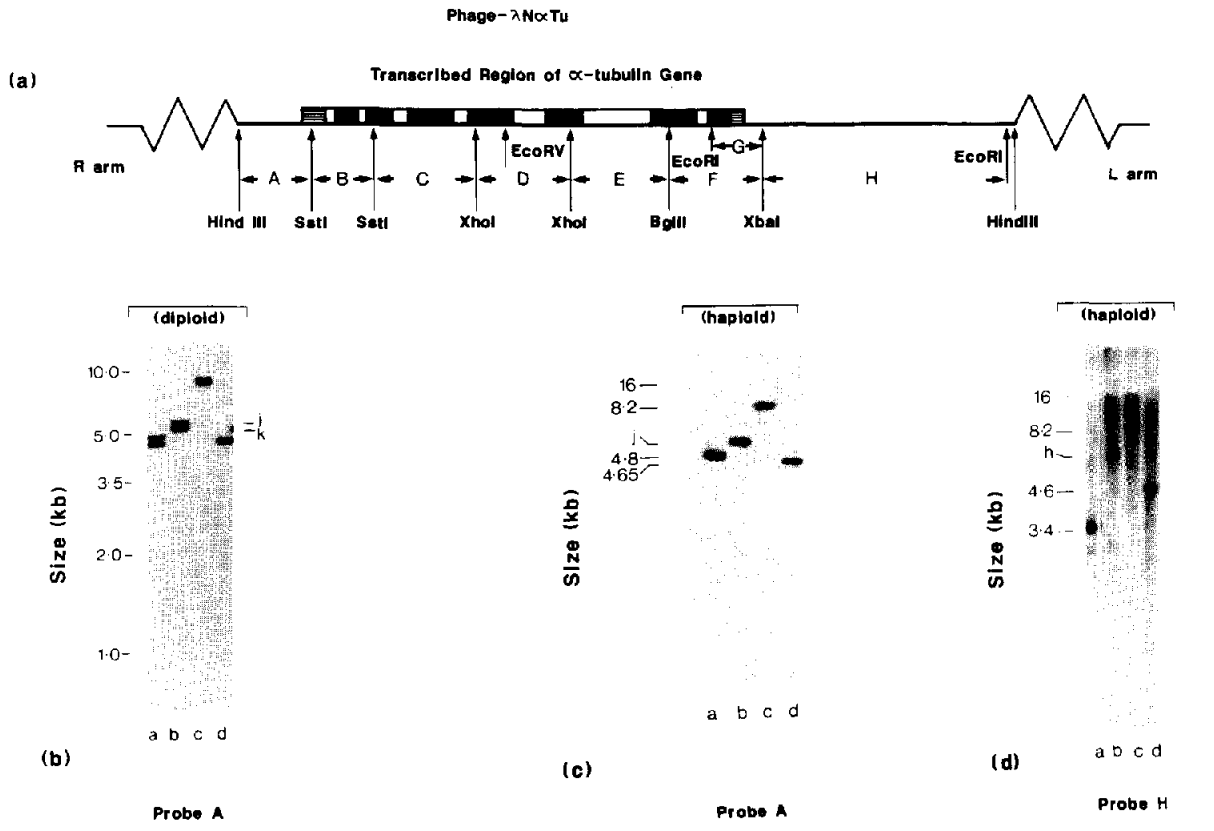

Fig.1. Hybridisation of ^{32}P-labelled sub-fragments of the *Nα*-tubulin gene with *Physarum* plasmodial (M_3CVIII) and amoebal (CLdAXE) DNA: (a) restriction map and structural features of the *Nα*-tubulin gene [6]. R arm and L arm respectively refer to the right and left arms of the vector (phage-λ NM1149). The probes A–H are shown beneath. (b) Gene-specific probe A recognises the 5′-ends of two forms of the *Nα*-tubulin gene in diploid M_3CVIII DNA, fragments j and h. (c) Probe A recognises 5′-end fragment j in haploid CLdAXE DNA. (d) Gene-specific probe H recognises 3′-end fragment h of the *Nα*-tubulin gene in haploid CLdAXE DNA. Digests in b–d are: lane a, *Eco*RV/*Kpn*I; lane b, *Eco*RV; lane c, *Kpn*I; lane d, *Hin*dIII.

sequence of the transcribed region of the *Nα*-tubulin gene is very similar, if not identical, in both cases; however, the alleles differ in the sequences flanking the 4.65 kb *Hin*dIII unit, possibly by insertions/deletions (see fig.3). A comparable situa-

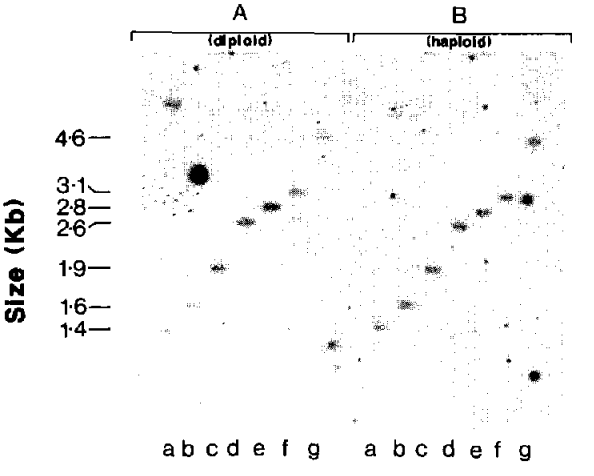

Fig.2. The transcribed regions of the two alleles of the *Nα*-tubulin gene are very similar in structure. (A) Plasmodial (M_3CVIII) and (B) myxamoebal (CLdAXE) DNA were digested with pairs of enzymes viz (a) *Hin*dIII/*Xho*I; (b) *Hin*dIII/*Eco*RV; (c) *Hin*dIII/*Stu*I; (d) *Hin*dIII/*Bgl*II; (e) *Hin*dIII/*Eco*RI; (f) *Hin*dIII/*Xba*I; and (g) *Hin*dIII alone. Gene-specific probe A was used for hybridisation. Previous experiments established that the 450 bp probe hybridised to a 450 bp fragment of *Physarum* DNA digested with *Hin*dIII/*Sst*I (not shown).

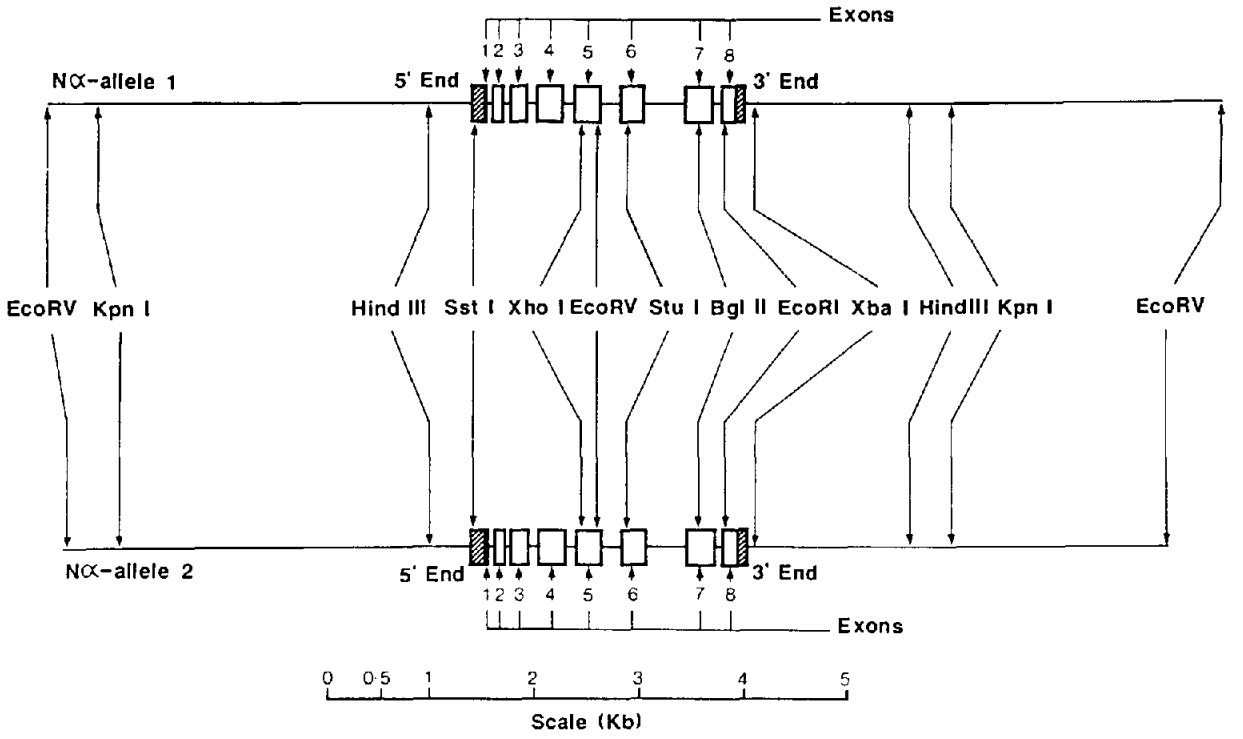

Fig.3. A summary of the structure of the two alleles of the *Nα*-tubulin gene. The map was constructed from data presented in figs 1 and 2. The structure of the transcribed region of the cloned *Nα*-tubulin gene (fig.1a) is assigned to each of the alleles on the basis of their identical internal restriction maps. The 5′- and 3′-nontranslated regions of the gene-transcripts are hatched. The map is drawn to scale, within the limits of accuracy imposed by agarose gel electrophoresis. Allele 1, which comprises fragments h and j, is found both in haploid CLdAXE DNA and in diploid M_3CVIII DNA. Allele 2, which comprises fragments i and k, is the second allele found in diploid M_3CVIII DNA.

tion was reported for the ardC actin gene of *Physarum*. The ardC2 allele was found to differ from the ardCl allele by the deletion of 700 bp near the polyadenylation site of the C-actin gene [12]. Formally, a segregation analysis of the two putative alleles is required to exclude mechanisms such as gene duplication for yielding two forms of the *Nα*-tubulin gene in the diploid strain M_3CVIII. However, the simplest and most probable explanation is that the strain M_3CVIII is heterozygous, particularly since one of the putative alleles has been identified in the haploid strain CLdAXE (see table 1).

Table 1

*Eco*RV fragments assigned to *Nα*-tubulin gene sequences (see fig.1)

	Haploid strain CLdAXE	Diploid strain M_3CVIII
5′-fragment(s)	j (5.4 ± 0.1 kb)	j,k (5.0 ± 0.1 kb)
3′-fragment(s)	h (6.2 ± 0.25 kb)	h,i (5.5 ± 0.1 kb)

3.4. *Summary*

In brief, we have used gene-specific probes to identify the *Eco*RV fragments of DNA of the haploid strain CLdAXE which span the *Nα*-tubulin gene. An identical pair of fragments were located in digests of DNA of the diploid strain M_3CVIII. Two other fragments were also identified in this digest indicating the presence of a second allele. However, the coding sequences of the putative alleles are contained in similar 4.65 kb *Hin*dIII fragments.

Acknowledgements: We wish to thank Mr S. Kotecha and Mr A. Stewart for their skilled technical assistance.

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