Subcloning of the Histone DNA Sequences of Phage Lambda Sam 7 h 22 in Plasmid pBR 322

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Sea Urchin Histone DNA, pBR 322, Subcloning

The histone DNA sequences of the Hind III cluster of the sea urchin *Psammechinus miliaris* which are carried by the chimeric phage DNA of λ Sam 7 h 22 have been subcloned in plasmid pBR 322. Due to this procedure single segments of the cluster have been separated from each other and are available as gene specific hybridization probes.

Introduction

The purification and characterization of histone mRNA is a relatively straightforward procedure if conducted with the RNA from early cleavage stage sea urchin embryos [1–6] where histone mRNA is the most abundant mRNA component found in small polysomes [1, 2]. Consequently this has led to an extensive characterization of the different subtypes of histone mRNA [4–9] as well as to their use as hybridization probes to detect and characterize cloned histone DNA sequences from various sea urchin species [10–13].

Obtaining pure histone mRNA from other sources is, however, a difficult and tedious task. Therefore, the characterization of the histone genes in these systems either by in situ hybridization [14–17] or by cloning of genomic DNA fragments has been based mainly on the use of the heterologous sea urchin histone mRNA or cloned sea urchin histone DNA as hybridization probes [18].

Sea urchin histone DNA is basically organized in regular tandemlike clusters containing all five histone genes. Within this basic repeat is contained an excess of spacer DNA [9]. Therefore, the original cloning experiments have led to cloned clusters instead of to individual histone genes. However, when analyzing differential histone mRNA expression in a given organism it is desirable to have hybridization probes which are specific for each gene. This is also true when analyzing whether a cloned histone specific genomic DNA sequence contains more than one of the five histone genes. To cleave the cloned sea urchin histone DNA clusters with the appropriate restriction enzymes and to purify the fragments on a preparative scale is technically unrewarding and does not lead to satisfactory purity if fragments of similar sizes have to be separated. A more satisfying approach resides in subcloning the segments of the cluster, thereby obtaining the desired fragments in sufficient purity and quantity.

We among others became interested for the reasons stated above [19] in obtaining subcloned histone DNA sequences from the sea urchin *Psammechinus miliaris*. As a vector the plasmid pBR 322 [20] was chosen as it did not impose restrictions on the clonable size of the histone sequences. This paper will describe the subcloning of several DNA sequences derived from the main histone DNA cluster of *P. miliaris* contained within the hybrid phage λ Sam 7 h22.

Materials and Methods

Restriction endonucleases Hind II, Hind III, Eco RI and PstI were from Boehringer, Mannheim. Bam H1 and Hae III were purchased from Miles. The procedures for plasmid purification, restriction enzyme digestion, phosphorylation with [γ^{32}P]ATP and polynucleotide kinase, ligation of Hind III linker and agarose gel electrophoresis have been detailed [21]. The hybrid phage λ b538 redB113 imm^{434} Sam 7 (Pm histone 22), abbreviated λ Sam 7 h22 was the kind gift of Dr. M. Birnstiel, University of Zürich. The phage was grown as published [10]. For transformation with plasmid DNA *E. coli* strains K12 490 *recA*$^-$, $r_k^- m_k^-$ *met*$^-$ *thr*$^-$ *lac y*$^-$ (obtained from Dr. G. Hobom, University of Freiburg) or HB 101 *F*$^-$ *pro*$^-$ *leu*$^-$ *thi*$^-$ *lac y*$^-$ *Str*^{r} $r_k^- m_k^-$ *endo R*$^-$ *recA*$^-$ were

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used. Transformants were grown on L-plates [22] containing 100 μg/ml ampicillin and tested on plates containing 5 μg/ml tetracycline. Hybridization of DNA fragments with ^{32}P-labelled 6 kb DNA after transfer to a cellulose nitrate sheet [23] was as described [24].

All subcloning procedures were performed in a P2 facility according to the German Guidelines for the Safe Handling of Recombinant DNA.

Results

Cloning of a H4 gene specific DNA fragment

The sea urchin 6 kb histone DNA cluster which is contained in the hybrid phage λ Sam 7 h22 is delineated by two half Hind III sites and contains one internal Eco RI site (25, 26, Fig. 1). The Hind III sites have been used to integrate the 6 kb histone DNA cluster into the vector phage [10]. When cleaving 6 kb DNA with Eco RI a DNA fragment is generated which contains the H4 cistronic DNA sequence, part of the Eco RI spacer and a short sequence out of the Hind III spacer [25, 26]. There is, however, no DNA sequence present in this fragment which will hybridize with the RNAs from the other 4 histone genes [25]. To subclone this DNA fragment the λ Sam 7 h22 DNA was digested with Hind III and, without prior purification of the 6 kb DNA, with Eco RI and ligated to pBR 322 DNA (Fig. 2) which had been cleaved with these two enzymes. The 29 bp Hind III/Eco RI fragment of pBR 322 [27] had been removed prior to ligation by sucrose density gradient centrifugation to prevent recircularization of the plasmid DNA under the ligation conditions.

If the 6 kb DNA is not purified from the vector sequences after Hind III digestion before Eco RI cleavage two more fragments are generated from the vector DNA which are terminated with a Hind III site on one and an Eco RI site on the other end and will be integrated into the plasmid DNA. After transformation with this ligation mixture ampicillin resistant transformants were analyzed by a minilysate procedure [21, 28] including agarose gel electrophoresis of the Eco RI/Hind III cleaved plasmid DNAs. Out of 24 independent clones picked at random and analyzed by this procedure 5 contained the H4 specific DNA fragment (clones pGPMh4, *cf.* Fig. 4, lane 19, and as molecular weight reference,

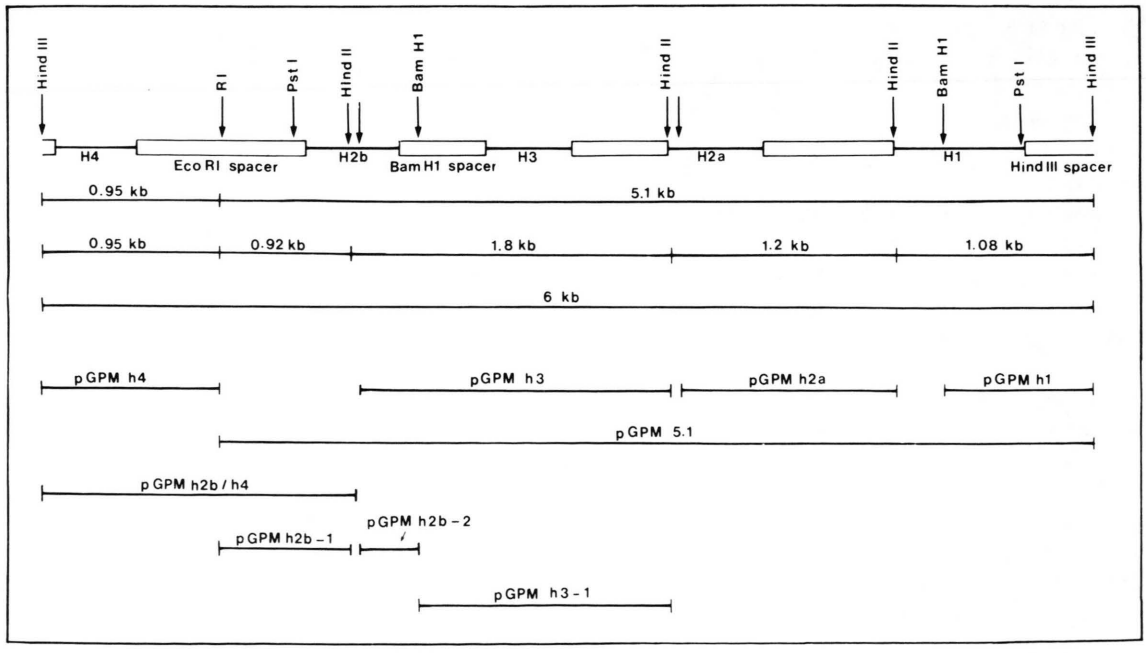


Fig. 1. Restriction map of the histone DNA cluster of λ Sam 7 h 22 [25, 26]. The relative positions of the subcloned DNA segments and the designation of the clones are given. The fragment lengths quoted above are used throughout this paper to designate the different fragments though slightly different sizes have been determined in this paper. Cistronic DNA sequences of the histone DNA cluster are indicated by thin lines, spacer sequences by blocks [26].

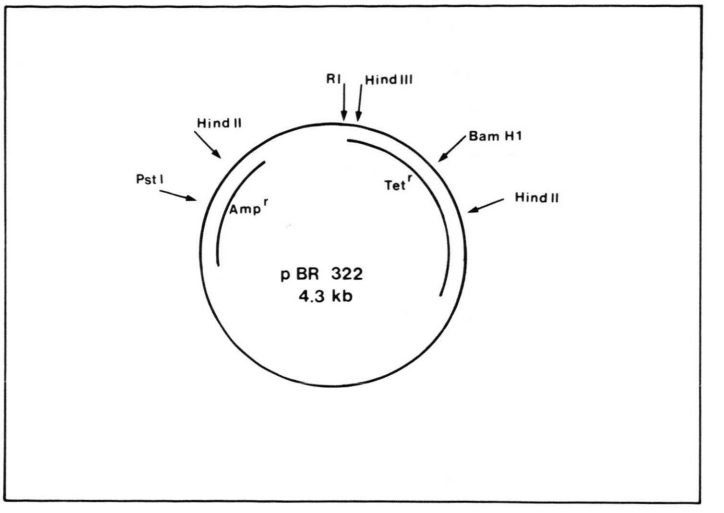


Fig. 2. Schematic outline of plasmid pBR 322 and the restriction sites relevant for the subcloning experiments described in this paper.

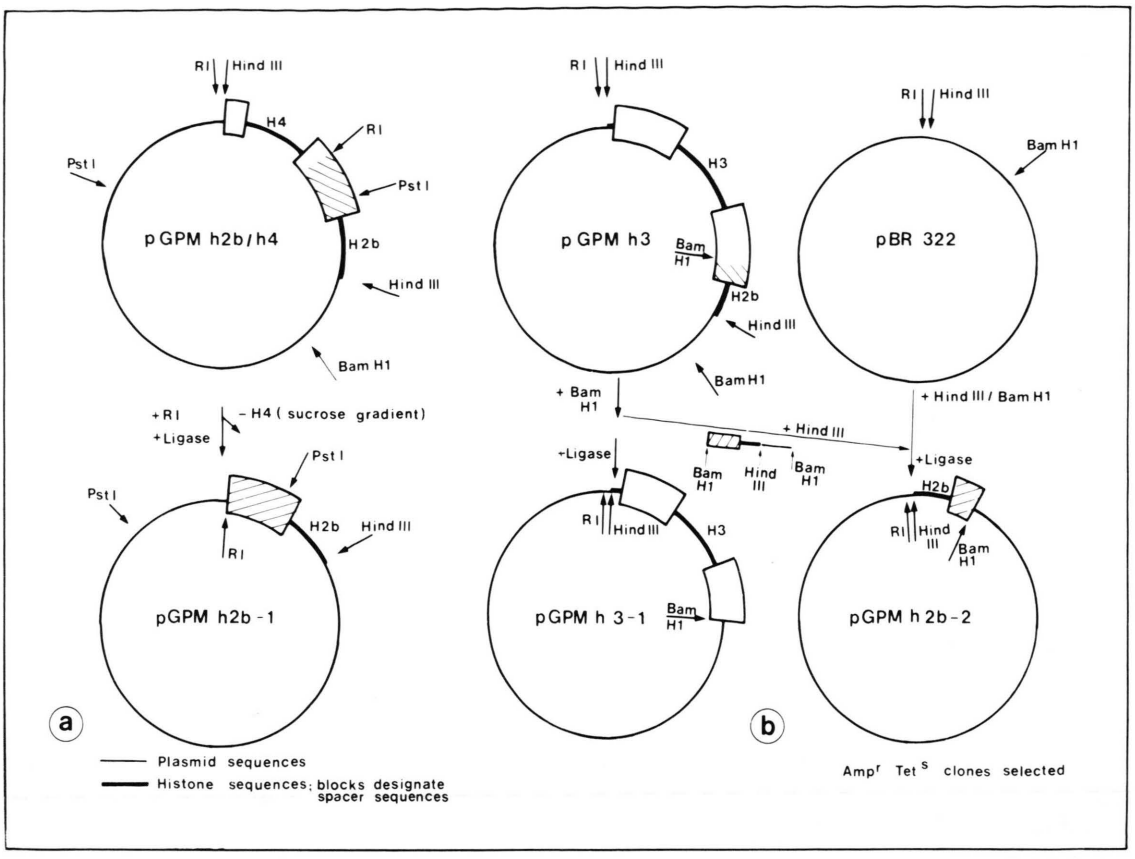
lane 18), and 4 the 5.1 kb fragment (clones pGPM5.1) which represents the *P. miliaris* histone DNA cluster lacking the H4 specific fragment. The remainder of the clones contained either vector specific sequences of unidentified inserts.

Cloning of a H2a specific DNA fragment

Another DNA fragment which may be obtained as a nearly full length cistronic DNA plus spacer DNA but with almost no contribution by another cistronic DNA sequence is the 1.2 kb Hind II fragment of 6 kb DNA [25, 26]. This fragment contains the coding sequence for the H2a gene. The 1.2 kb DNA cannot be integrated directly into pBR 322 DNA as the latter has 2 Hind II sites (Fig. 2) and will yield a complex and inefficient ligation. As the 1.2 kb Hind II fragment has blunt ends [29] it can be coupled, however, to chemical linker oligomers containing the recognition site for *e. g.* Hind III [30] which cuts pBR 322 only once (Fig. 2). According to this strategy *P. miliaris* 6 kb histone DNA was obtained by Hind III digestion of λ Sam 7 h22 DNA, agarose gel electrophoresis and elution from the gel including purification on a CsCl density gradient [10]. It was cleaved with Hind II and ligated to the double-stranded Hind III specific linker decamer [31–33]. After Hind III digestion to expose the internal Hind III specific cohesive ends of the linker, ligation to Hind III cleaved pBR 322 and transformation, ampicillin resistant clones were checked for the presence of histone DNA specific clones by a colony hybridization procedure [34, 35] using a ^{32}P-labelled cRNA from λ Sam 7 h22 DNA as a probe [36]. This selection step identified all those clones which contained a hybrid plasmid derived from 6 kb DNA and pBR 322 DNA. The histone DNA specific inserts which are cleaved from the plasmid DNAs by Hind III digestion were shown to be identical in size with those arising after Hind II digestion of 6 kb DNA. They were identified by electrophoresis on agarose gels and hybridization with ^{32}P-labelled [37–39] 6 kb DNA after transfer to a cellulose nitrate sheet [23]. Of 9 independent 6 kb DNA derived clones one contained the desired H2a sequence (Fig, 4, lane 17, clone pGPMh2a), 4 the H2b/H4 specific Hind III/Hind II bounded fragment (lane 15; clone pGPMh2b/h4) and 4 the H3 + H2b specific Hind II bounded DNA fragment (lane 16; clone pGPMh3, ref. [25, 26]). None was found which contained the H1 specific Hind II/ Hind III delineated 1.08 kb [25] DNA sequence.

Cloning of two H2b specific DNA fragments

Subcloning the Hind II fragments of 6 kb DNA served to divide the histone DNA into four major sequence blocks. No subclone was expected by this procedure to contain the H2b sequence alone (Fig. 1). Part of the H2b cistronic DNA may, however, be subcloned from a H4/H2b containing clone by Eco RI digestion and recircularization of the linearized vector DNA if it contains the DNA insert in the appropriate orientation. In this case Eco RI diges-



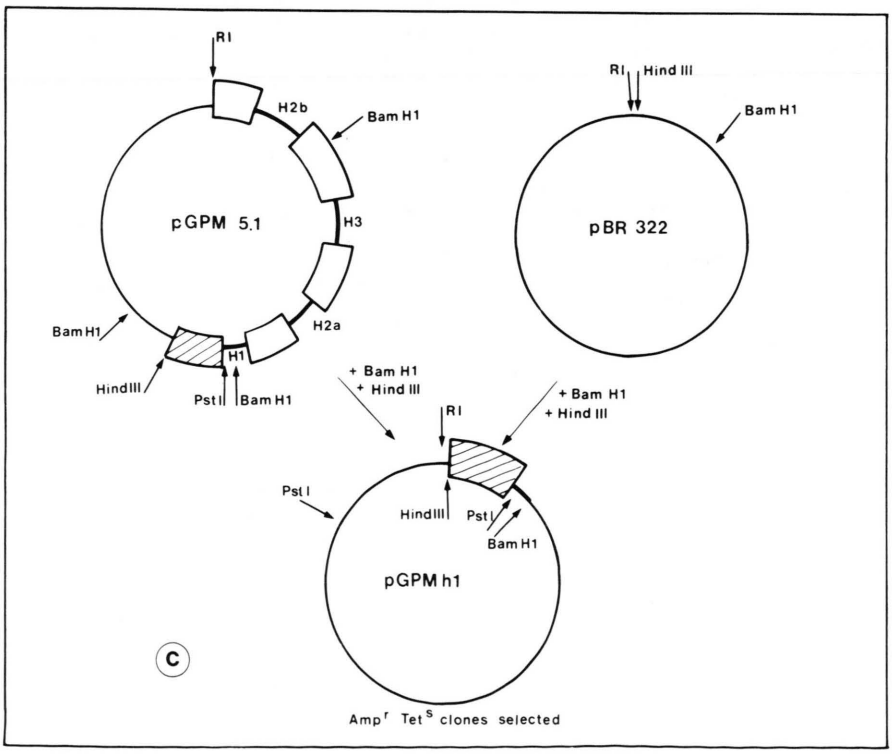


Fig. 3. Scheme of the strategy used for subcloning some of the histone DNA fragments.

tion leads to the release of the H4 fragment. Two hybrid plasmids containing the former 1.87 kb Hind II/Hind III fragment (Fig. 1) were analyzed for the orientation of the insert using the internal Pst I site as a marker [26]. One (pGPMh2b/h4) was found to be in the appropriate orientation (Fig. 3a) as it had the Pst I site in the insert adjacent to the Bam H1 site of pBR 322. It was cleaved with Eco RI and purified from the smaller H4 specific fragment by sucrose density gradient centrifugation. It was treated with DNA ligase and used for transformation. The plasmid DNA from one ampicillin resistant transformant (pGPMh2b-1) was shown by Hind III and Eco RI cleavage to contain the former Eco RI/Hind II bounded H2b specific ("0.92 kb") DNA fragment (*cf.* Fig. 4, lane 12).

From Fig. 1 it is obvious that almost all of the lacking part of the H2b cistronic sequence may be derived from a clone (pGPMh3) containing the H3 gene specific Hind II bounded fragment thereby also generating a subclone specific for H3 cistronic DNA including the flanking spacer sequences. The hybrid plasmid of clone pGPMh3 contains the former Hind II fragment in an orientation where the internal Bam H1 site is proximal to the Bam H1 site of pBR 322 (Fig. 3b). This was shown by 5′ end labelling [40] after Eco RI digestion of the plasmid DNA and an ensuing Bam H1 digestion. The plasmid DNA was therefore cleaved with Bam H1, the smaller fragment was purified on a sucrose density gradient, cleaved with Hind III and used for ligation with Bam H1/Hind III digested pBR 322 DNA.

Table I. Comparison between fragments of 6 kb DNA and the histone DNA plasmid subclones after digestion with various restriction enzymes.

Lane	6 kb Hind II/ Pst I (a) 1	6 kb Hind II/ Bam H1 3	6 kb Bam H1/ Pst I (a) 5	6 kb Hind II/RI 11	6 kb Hind II 14	6 kb RI 18	pGPMh2b−1 Pst I (a)/Hind III 2	pGPMh2b−2 Hind III/Bam H1 4	pGPMh3−1 Bam H1/Hind III 6	pGPMh1 Hind III/Bam H1 7	pGPMh1 Bam H1/Pst I (a) 8	pGPMh2b−1 RI/Hind III 12	pGPMH2b/h4 Pst I (a) 13	pGPMh2b/h4 Hind III 15	pGPMh3 Hind III 16	pGPMh2a Hind III 17	pGPMh4 Hind III/RI 19
						n. d.		(4.0)	(4.0)	(4.0)		(4.3)		(4.3)	(4.3)	(4.3)	(4.3)
													4.0				
							(3.5)										
											(3.1)						
			3.0														
													2.4				
		2.05			2.0									2.0			
	1.93			1.95	1.9										1.9		
	1.66		1.65														
		1.60							1.6								
							1.45										
											1.37						
	1.29	1.34		1.25	1.3											1.3	
				1.1	1.15												
				0.99		1.1											1.1
				0.94								0.99					
		0.89								0.86							
			0.76														
			0.60														
	0.58																
	0.43						0.43										
		0.36						0.36									
	n. d.		0.34								0.33						
		0.34															

The values are taken from Fig. 4. Fragment lengths in kb. Fragments, the lengths of which are written on the same line, have been derived from identical locations within the histone DNA cluster. ( ): Pure plasmid sequence. ——— : Fragment contains plasmid sequences and histone DNA; (a): Pst I digestions tended to yield some partial digestion products; this does not, however, influence the interpretation of the data.

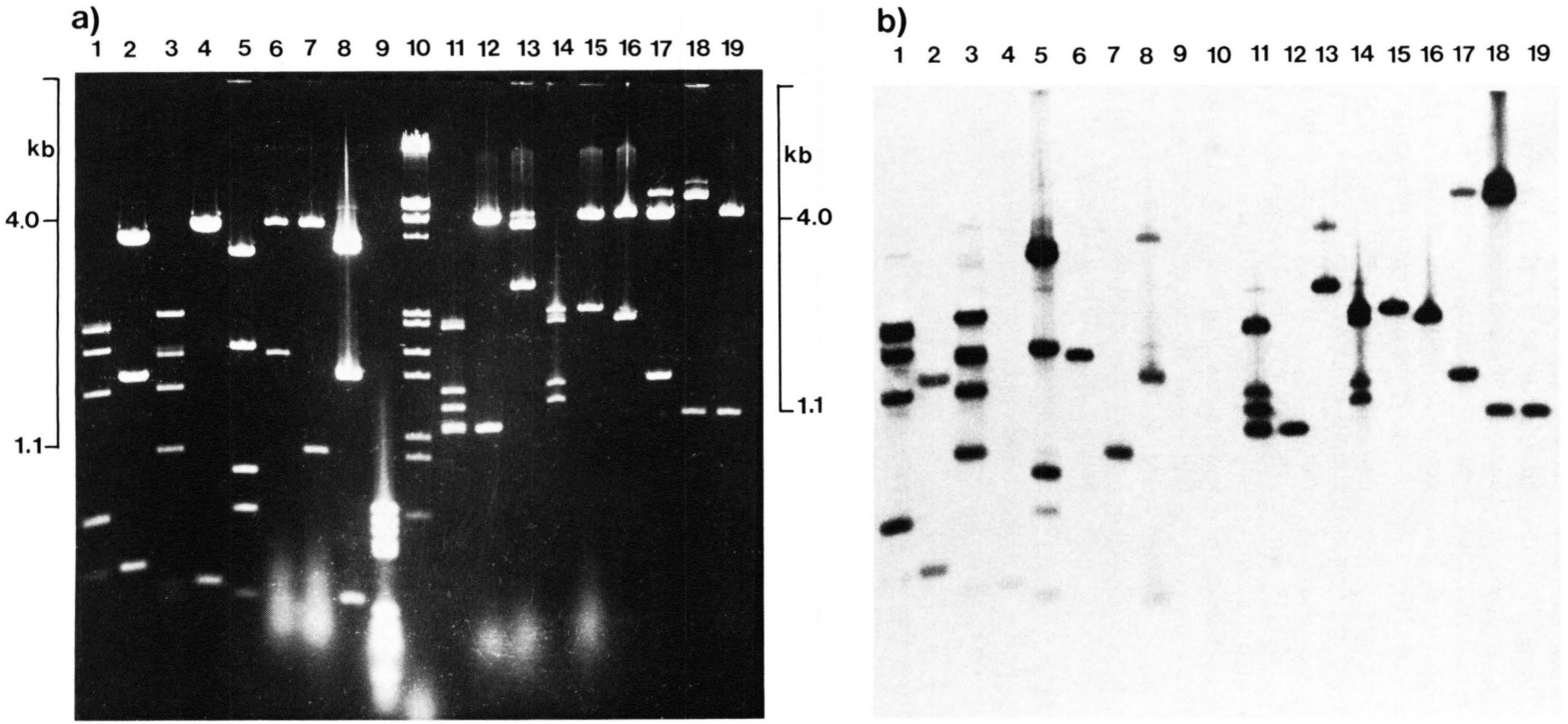


Fig. 4. Restriction analysis of the histone DNA subclones after transfer to pBR 322. 6 kb DNA and the various histone DNA containing hybrid plasmids were purified on a preparative scale and cleaved with the restriction enzymes specified below. After fractionation on a horizontal 1% agarose gel fragments were photographed under UV light after ethidium bromide staining (a). The DNA fragments were transferred to a cellulose nitrate sheet and hybridized with 6 kb ^{32}P-labelled histone DNA to identify the histone DNA specific plasmid inserts (b). Fragment lengths are given in Table I. Restriction fragments came from the following digestions: (1) 6 kb DNA Hind II/Pst I, (2) pGPMh2b-1 Pst I/Hind III, (3) 6 kb DNA Hind II/Bam H1, (4) pGPMh2b-2 Hind III/Bam H1, (5) 6 kb DNA Bam H1/Pst I, (6) pGPMh3-1 Bam H1/Hind III, (7) pGPMh1 Hind III/Bam H1, (8) pGPMh1 Bam H1/Pst I, (9) pBR 322 Hae III, (10) λ DNA Eco RI/Hind III, (11) 6 kb DNA Hind II/Eco RI, (12) pGPMh2b-1 Eco RI/Hind III, (13) pGPMh2b/h4 Pst I, (14) 6 kb DNA Hind II, (15) pGPMh2b/h4 Hind III, (16) pGPMh3 Hind III, (17) pGPMh2a Hind III, (18) 6 kb DNA Eco RI, (19) pGPMh4 Hind III/Eco RI. Fragment lengths of the marker DNAs are taken from (27) and (45–46). The restriction fragments in lanes 1, 3, 5, 11, 14 and 18 have served for the identification of the histone DNA specific inserts in the hybrid plasmid DNAs (*cf.* Fig. 1).

After transformation 4 out of 10 ampicillin resistant transformants were shown to be tetracycline sensitive and to contain in their plasmid DNAs a 360 bp Bam H1/Hind III fragment which hybridizes with labelled 6 kb DNA (Fig. 4, lanes 3 and 4). According to the published data [24, 25] this fragment represents the "right hand" part of the H2b gene (Fig. 1).

The large fragment arising from the Bam H1 digestion of plasmid pGPMh3 was self-ligated after sucrose gradient purification. Upon transformation it yielded ampicillin resistant clones the plasmid DNAs of which contained a 1.6 kb Bam H1/Hind III fragment in all ten transformants tested thus (clone pGPMh3−1). This fragment represents the H3 cistronic DNA together with the two flanking spacer sequences (Fig. 1, Fig. 4, lane 6).

Cloning of a H1 specific DNA fragment

Among the clones arising from the Eco RI and Hind III digestion of λ Sam 7 h22 DNA (see above) one was obtained (pGPM5.1) which contains the 5.1 kb Eco RI/Hind III fragment of 6 kb DNA in pBR 322. There is only one possible orientation of this fragment in the plasmid DNA. As outlined in Fig. 3c, cleavage of the plasmid DNA of this clone (pGPM5.1) with Hind III and Bam H1 will generate two Bam H1/Hind III bounded fragments which can be integrated into likewise cleaved pBR 322 DNA. Transformation with these plasmid DNAs will yield among others Amp^r Tet^s clones which will harbor a H1 cistronic DNA fragment plus part of the Hind III spacer (Fig. 1). According to this outline plasmid pGPM5.1 was cleaved with Bam H1 and Hind III and the three small fragments arising were separated from the linear residual plasmid DNA by sucrose density gradient centrifugation and ligated to Hind III/Bam H1 cleaved pBR 322 DNA. Ampicillin resistant tetracycline sensitive clones were grown up and after purification their plasmid DNAs were shown by Bam H1 and Hind III digestion to contain a 0.86 kb DNA insert which hybridizes with labelled 6 kb DNA (Fig. 4, lane 7). Bam H1 and Pst I digestion of the plasmid DNA yields two hybridizable fragments of 1.37 and 0.33 kb (Fig. 4, lane 8).

To demonstrate the authenticity of the subcloned DNA fragments two of them (pGPMh3 and pGPMh2a) were mapped by partial restriction with Hae III within the histone gene regions (data not shown). Though there were some differences in size compared to the published values [26, 40] the agreement in size was good and there was no difference in the number of the cleavage sites. From this it may be concluded that these and − with a high likelihood − also the other subcloned histone DNA sequences are authentic and have not been changed by the scrambling of sequences during the subcloning procedures.

Discussion

The histone gene subclones described in this paper may be roughly divided into three groups two of which are monospecific for single histone genes and therefore are particularly interesting. Of these the first consists of those clones which contain most or all of a histone specific cistronic DNA but with significant contributions by spacer DNA. They will be useful tools as hybridization probes if expression of individual histone genes is to be studied. Examples are the clones pGPMh4, pGPMh3−1, and pGPMh2a. The second group consists of those clones where the plasmid DNA carries only part of the cistronic DNA but from which the major part of the spacer sequences can be released by cleavage with another restriction enzyme if necessary. They will be equally useful for the study of single gene expression but their major advantage is expected in those cases where crosshybridization of the spacer sequences might lead to an erroneous interpretation of hybridization experiments. Examples for this group are the clones pGPMh2b−1 after Hind III and Pst I or Hinf 1 digestion and pGPMh1 upon Bam H1 and Pst I cleavage [26].

During the progress of this work various other subclones of sea urchin histone DNA clusters have been obtained by other groups [41−44]. These and the subclones described in this paper will constitute valuable tools in further investigations into the structure and expression of the histone genes of sea urchins as well as of other organisms.

Acknowledgements

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