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Molecular cloning and characterization of small polydisperse circular DNA from mouse 3T6 cells

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ABSTRACT

We have isolated, cloned and analyzed small polydisperse circular (spc) DNA from mouse 3T6 cells. The representation of highly repeated mouse genome sequence families in spcDNA has been examined, and the B1 repeat appears overrepresented in spcDNA by two criteria. The majority of spcDNA clones, however, is made out by as yet uncharacterized middle repetitive sequences. We have investigated the increase in the spcDNA population upon cycloheximide treatment of individual sequences, which are found to amplify differentially.

INTRODUCTION

In addition to chromosomal and mitochondrial (mt) DNA, mammalian cells contain small polydisperse circular DNA. These extrachromosomal DNA circles of varying sizes have been identified in amounts ranging from 0.001 to 0.1 % of total DNA. They are present in all tissues and cell lines of mammalian, avian and insect origin that have been examined (1-5). spcDNA is a heterogeneous population of sequences, and in contrast to plasmids, there is no fixed copy number. Rather, the number of spcDNA molecules per cell increases as a result of a variety of stimuli, such as treatment with protein synthesis inhibitors or cultivation in stationary phase. Since spcDNA is homologous to chromosomal DNA, excision from chromosomes and circularization of cDNA from reverse transcription are considered the main sources of spcDNA, although limited episomal replication has not been excluded. Current hypotheses on the formation of spcDNA include "replicon misfiring" (3,6,7) and as intermediates in transposition (8,9). In certain developmental stages, specific genomic rearrangements may leave circular DNA molecules as a byproduct (5,10).

The main part of work on mammalian spcDNA on the molecular level

has been carried out in primate cells such as HeLa (3,11) or BSC-1 (2,9,12). There is growing evidence that there are individual sequences represented in spcDNA as discrete species, indicating that spcDNA formation is not a random event. Thus, Schindler and Rush (12) demonstrated that Kpn I repeats show as distinct size classes in BSC-1 spcDNA and Paulson et al. (8) showed that the middle repetitive element THE 1 hybridizes to discrete molecular lengths in spcDNA from an established human cell line.

Our previous electron microscopic studies (13) indicated the possibility that transfer of foreign DNA to mammalian cells provokes an increased production of (cellular) spcDNA. This effect was demonstrated in several cell lines, but most of our work was done with mouse 3T6 cells. We want to settle this issue, and also study more general questions regarding spcDNA, such as their mechanism of induction and possible involvement in recombination and amplification of DNA. As a first step we have isolated, cloned and characterized spcDNA from mouse 3T6 cells.

MATERIALS AND METHODS

Cell culture. Mouse 3T6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml and 100 µg of streptomycin per ml.

Purification of cccDNA for cloning. Mouse 3T6 cells, 10^9 at the time of harvest, were grown to near confluence. At 24 h before harvest ^{3}H-thymidine was added to a 5% aliquot of the cells. 16 h before harvest, cycloheximide was added to a final concentration of 50 µg/ml to all cells. Circular DNA was purified by alkaline denaturation essentially as described (4), but cccDNA was purified on three successive CsCl-ethidium bromide (EtBr) density gradients. Fractions from the third gradient containing circular DNA were pooled, destained, ethanol precipitated and dissolved in TE. The purity of the final preparation was examined by electron microscopy.

Electron microscopy. Visualization of DNA with an aqueous droplet technique and measurement of contour lengths using mouse mtDNA (16295 base pairs <14>) as a length standard have been described earlier (15).

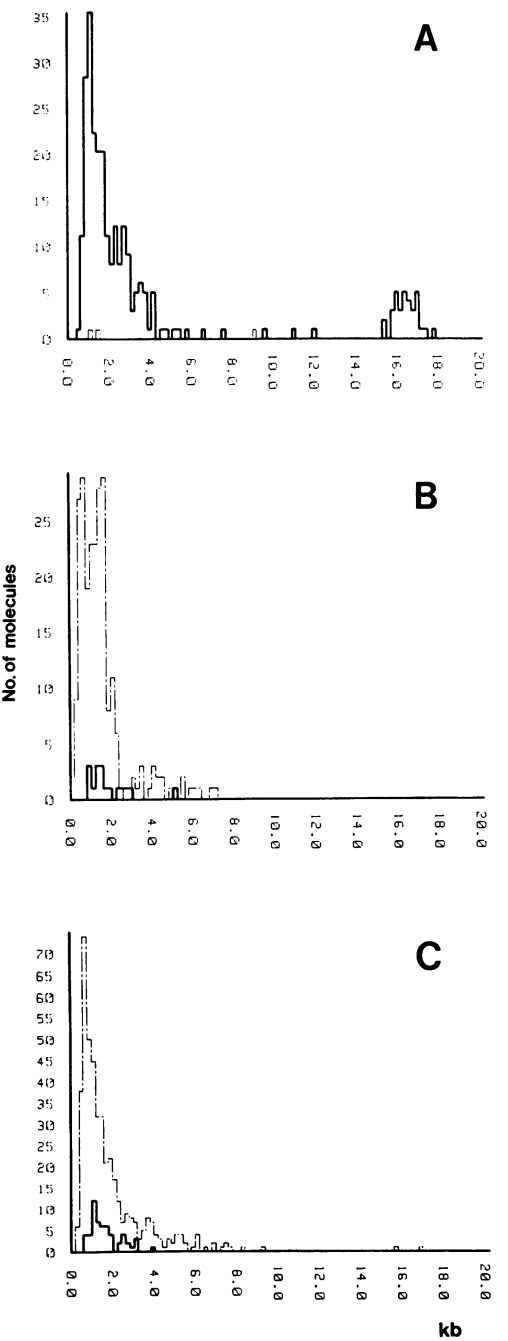
Construction of spcDNA clone libraries. **The four-cutter library.** Aliquots, 50 ng each, of the cccDNA preparation were digested separately with MspI or Tth HB 8I (an isoschizomer of TaqI). The DNA fragments were made blunt end with T4 DNA polymerase and ligated to Bam HI- and PstI-linkers. After cleavage of linkers and separation on Sepharose CL-6B, a portion (10 ng) was ligated to 25 ng of Bam HI-, Pst I- double cleaved pUC 9 (16). E. coli RR1 were transformed to ampicillin resistance by the procedure of Hanahan (17), and recombinant clones were identified by scoring Lac Z phenotype on plates containining Xgal. MtDNA clones were eliminated by colony hybridization using the gel-purified inserts of cloned mouse mtDNA Bam HI fragments 1 through 4 (G. Bjursell, unpubl., 14) as probes.

The six-cutter library. A 100 ng portion of the cccDNA preparation was digested with Kpn I, Xma I and Sal I and thereafter divided into six aliquots. The first aliquot was digested with Cla I, no. 2 with Pst I, no. 3 with Bgl II, no. 4 with Hae II plus Sac I, no. 5 with Hae II plus Sph I, and no. 6 with Xho I. The aliquots were pooled, and Bam HI- and Pst I-sites were protected by methylation. Addition of Bam HI- and Pst I-linkers, ligation to pUC 9, transformation of RR1 and screening of colonies were as described for the four-cutter library.

Radiolabeling of DNA. Whole plasmids were labeled by nick-translation (18). Inserts of clones, purified by gel electrophoresis followed by electro-elution, total 3T6 DNA or total cccDNA were labeled using "oligo-labeling" (19).

Southern blotting. Total 3T6 DNA was prepared essentially as described (20). Electrophoresis was carried out in 1% agarose gels in Tris-borate buffer. After acid treatment, the DNA was transferred (21) to nitrocellulose filters.

Gradient slot blotting of DNA from control or cycloheximide-treated cells. 3T6 cells, 10^8, were grown to about half saturation density. Labeling of DNA with ^{3}H-thymidine and cycloheximide treatment were as described above. DNA from Hirt supernatants (22) was centrifuged to equilibrium in CsCl-EtBr gradients. After fractionation, ^{3}H-radioactivity was determined and fractions containing circular DNA were pooled and


A
B
C
No. of molecules
kb
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0

recentrifuged. The second gradient was also fractionated, and fractions were stored in the dark until use. Aliquots were acid-treated, denatured and applied to nitrocellulose filters as described (4).

Hybridization. DNA, slot blotted, blotted from agarose gels or from bacterial colonies, after immobilization on nitrocellulose filters, was hybridized and washed as described (13).

DNA probes of highly repeated mouse DNA. Plasmid Mm 31 representing the B1 repeat family of the mouse genome (23) was provided by Dr. P. Fort. Plasmids pMR 142 and pMR 288, representing the B2 repetition (24,25) and the "evolutionarily conserved" (EC) sequence (24,26), respectively, were a kind gift of Dr. N. Hastie. A representative of the R group of sequences (27), R1/R2, was given to us by Dr H. Zachau. Dr. M. Meunier-Rotival furnished pMRB1-1 and pMRB5, together comprising most of the long mouse interspersed fragment (MIF) repeat (28). The inserts of the above clones were purified by gel electrophoresis. A mouse satellite containing plasmid, Clone 16 was provided by Dr. W. Hörz. After Taq I-, Hha I- and Eco R1-triple digestion and gel electrophoresis, a Taq I fragment encompassing 17 mouse satellite monomer repeats in tandem was recovered from this plasmid.

RESULTS

Preparation of cccDNA. In the third CsCl-EtBr gradient, better than 99% of the ^{3}H-radioactivity was found in the peak corresponding to circular DNA. Since the specific radioactivity after ^{3}H-thymidine labeling is identical in either chromosomal, mitochondrial or spcDNA, both before and after cycloheximide treatment (29), this figure is a valid estimate of the purity of cccDNA. Extensive electron microscopic examination reveals mtDNA

Fig. 1.
Length distributions, obtained by electron microscopy, of circular and linear DNA in the cccDNA preparation used for cloning before and after cleavage with restriction enzymes. Solid line, circular DNA; broken line, linear DNA.
A, native preparation; n = 268
B, four-cutter cleaved; n = 254
C, six-cutter cleaved; n = 498

molecules, spcDNA molecules of various sizes, and a few linear molecules. Fig. 1 A displays the size distribution of circular DNA in the cccDNA preparation. The number ratio of spcDNA to mtDNA molecules is 7 to 1, which equals a 70-fold amplification of spcDNA compared to 3T6 cells not treated with cycloheximide (13). The low amount of linear DNA, 1.5 %, is in agreement with the ^{3}H profile of the gradient. In close agreement with our earlier results for 3T6 cells not treated with cycloheximide (13), a mean size of 2.04 kilobase pairs (kb) is found for spcDNA. Thus, in contrast to the situation in Drosophila (30), the size of spcDNA does not decrease upon cycloheximide amplification.

spcDNA clone libraries. As the cloning protocol permits cloning of all contaminating linear DNA molecules, we found it necessary to estimate the proportion of circular DNA represented in the clone banks. This can be done by comparing the proportions of circular vs. linear DNA in the native and four-cutter and six-cutter digested cccDNA preparation (Fig. 1). It is noteworthy that short linear molecules (less than approx. 200 base pairs <bp>) are indistinguishable from the cytochrome c background. Hence, no conclusions can be drawn about the size distribution of the resulting clone inserts, which in fact have a considerably shorter mean length than suggested from Fig. 1 B and C (see Table 1). It is seen, however, that indeed the majority of spcDNA molecules have been linearized, at least 83% after four-cutter digestion and at least 78% after six-cutter digestion.

After elimination of mtDNA clones, 230 and 470 spcDNA recombinant clones remain in the four-cutter and six-cutter library, respectively. The properties of characterized clones are summarized in Table 1. The inserts of the four-cutter clones have a mean size of approximately 250 bp, and inserts up to 2 kb have been found. In the six-cutter library, insert sizes range from 200 bp to 3 kb, with a mean around 750 bp.

Representation of highly repeated sequence families in spcDNA clone libraries. Table 2 shows the number of clones in the two spcDNA libraries that are positive in colony hybridization with high stringency washing (see Materials and Methods) for highly

Table 1. Properties of individual spcDNA clones.

Clone (a)	Insert size (kb)	Homology with highly repeated DNA	Hybridization pattern in 3T6 DNA (b)	Stronger hybridization with cccDNA
	Four-cutter library			
pSPC-5	0.3	-	IS	
-8	0.4	B1	IS	
-20	2.0	-	B	+
-29	0.2	B1		
-31	0.1	MIF		
-47	0.5	-	IS	+
-49	1.0	B1		
-50	0.2	-	IS	
-52	0.6	-	IS	+
-53	0.2	B1		
-83	0.4	R		
-86	0.4	-	IS	+
-100	0.3	-	IS	
-102	0.5	-	IS+B	
-127	0.3	-	IS	
-145	2.0	-	IS+B	+
-165	0.2	-	IS	
-170	0.2	-	IS	
-174	0.3	-	IS	
-201	0.5	-	IS	
	Six-cutter library			
-238	1.0	-	IS	+
-243	0.8	-	IS	
-249	0.4	R		
-291	0.2	-	IS	
-292	1.3	B2		
-371	0.7	-	IS	
-372	0.3	B1		
-389	1.0	-	IS	+
-420	0.4	-	IS+B	
-492	0.3	-	IS	
-518	0.5	-	IS	
-537	0.2	MIF		
-547	0.2	-	IS	
-580	1.5	-	IS	+
-585	0.4	R	IS	
-612	0.5	R		
-621	0.8	B1		
-627	0.6	-	IS	+
-631	1.2	-	IS+B	
-641	0.2	-	IS	+
-644	1.0	-	IS	
-648	1.5	MIF		
-669	0.4	-	IS	
-679	1.7	MIF		
-688	0.6	-	IS	+

(a) Clones of which only insert size and lack of homology to highly repeated DNA or only homology to highly repeated DNA are known are not included in this table.
(b) B = one or several discrete bands
IS = hybridization smear characteristic of interspersed sequences

Table 2.
Representation of highly repetitive mouse DNA sequences in the spcDNA clone libraries.

Repeat element	No. of positive clones in spcDNA libraries				Fraction of chromosomal DNA (24,31)
	Four-cutter library	Six-cutter library	Total	%	
B1	10	25	35	5.0	0.9-1.3%
B2	-	2	2	0.3	0.5-0.8%
EC	1	5	6	0.8	0.5-0.8%
R	2	8	10	1.4	1.0-1.5%
MIF	4	10	14	2.0	4.0-5.0%
Satellite	-	-	-	-	5-10%

repetitive DNA. When stringency was reduced by lowering the hybridization and washing temperature to 48°C, identical results were obtained (data not shown).

When calculating the expected number of positive colonies, assuming a distribution identical to that of chromosomal DNA, the following has to be taken into consideration:

(i) When the cloned fragments are considerably longer than the repeated elements, the proportion of positive clones depends on the **repetition frequency** of the repeat elements. By contrast, if the cloned fragments are shorter than or not much longer than the repeat elements, the proportion of positive clones should equal the **fraction of the genome** constituted by a certain repeat element.

(ii) **The distribution of restriction sites** in the repeated elements. This dependence is stronger for long or tandemly arranged elements, since flanking sites will be farther apart than for short dispersed elements. The B1, B2 and R elements, in addition to being short (200 bp for B1 and B2, 400-500 bp for R <24>) and dispersed, all contain sites in their published prototype sequences contained in the EMBL database (23,25,27) for the enzymes used for cloning at a density comparable to that in total mouse DNA.

The five sequence families B1, B2, R, MIF and EC represent all dispersed sequences repeated more than 30 000 times per haploid

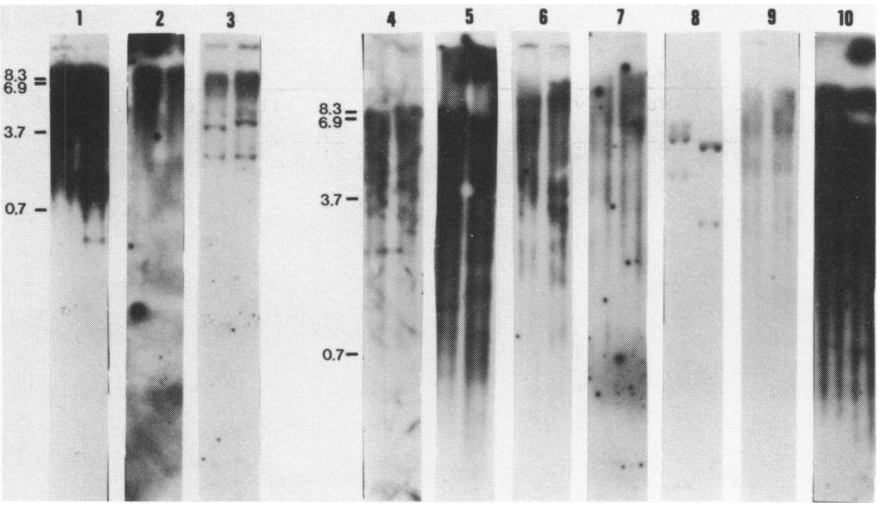


Fig. 2.
Southern blots of chromosomal 3T6 DNA probed with individual spcDNA clones. In each lane is shown to the left Bam H1- cleaved and to the right Eco R1-cleaved 3T6 DNA. Lanes 1 - 5, randomly chosen spcDNA clones. 1, pSPC-631; 2, pSPC-50; 3, pSPC-420; 4, pSPC-243; 5, pSPC-100. Lanes 6 - 10, spcDNA clones identified by differential hybridization. 6, pSPC-580; 7, pSPC-389; 8, pSPC-20; 9, pSPC-47; 10, pSPC-86.

genome (24). The only significant deviation from the expected number of colonies is for B1, which apparently is overrepresented about 5-fold. The EC prototype sequence (26) contain no sites for the enzymes used. Apparently cloning in flanking sites was sufficient to permit representation of this element near the expected frequency. The long (up to 7 kb <24>) MIF repeat has not been sequenced in its entire length, but examination of the sequenced parts reveals no drastic deviation from the average density of sites. A slight underrepresentation is found in both libraries for MIF-positive clones. The absence of satellite DNA from the clone banks was expected, since the prototype sequence (31) contains no sites for the enzymes used, although a minor fraction of satellite DNA is cleaved by Taq I (31). The tandem arrangement of most satellite sequences precludes cloning in flanking sites, and so no conclusions can be drawn from these data about the representation of satellite sequences in spcDNA.

Chromosomal organization of spcDNA clones. Fig. 2 shows Southern

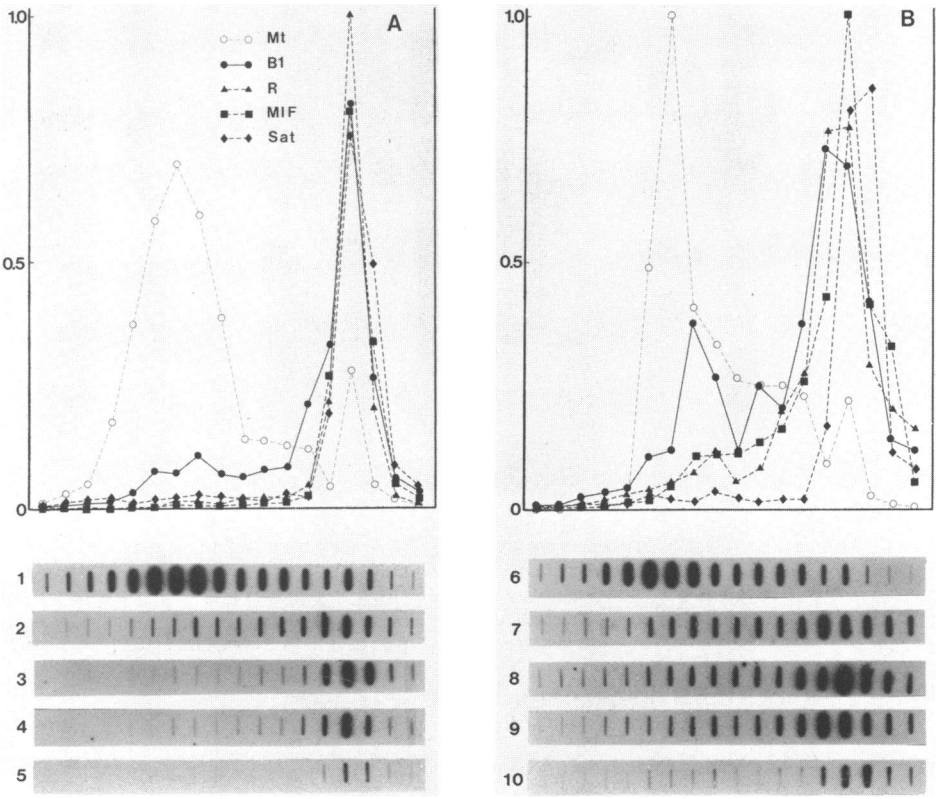


Fig. 3.
Hybridization of clones of highly repeated DNA and of mtDNA to CsCl-EtBr gradient slot blots of DNA from normal and cycloheximide-treated 3T6 cells, prepared as described in Materials and Methods. The fraction corresponding to the lower part of the tube is oriented to the left. After immobilization on nitrocellulose, the DNA was hybridized with the purified inserts of the following probes: Rows 1 and 6, mtDNA; 2 and 7, Mm31 (B1); 3 and 8, pMRB1-1 (MIF); 4 and 9, R1/R2 (R); 5 and 10, clone 16 (satellite). Rows 1 - 5, slot blots of one gradient from untreated cells; 6 - 10, of one gradient from cycloheximide-treated cells.
A, normal cells. The autoradiograms were scanned with an optical densitometer. Hybridization intensities were converted to absolute amounts of DNA using calibration curves prepared by applying total 3T6 DNA, sonicated, diluted in 47.8 % (w/w) of CsCl and 0.4 mg/ml of EtBr, and thereafter treated as above, in amounts ranging from 3 pg to 1 µg and hybridized with the same probe. The curves shown were normalized such that the sums of the DNA amounts of the fractions from the peak of linear DNA (fractions 14-16 from the left) were set equal. Relative DNA amounts are given on the Y-axis. For mtDNA, normalization was

instead done such that the sums of the DNA amounts from the circular peak (fractions 6-8 from the left) were equal in A and B. B, as in A but cells treated with cycloheximide.

blots where individual spcDNA clones were hybridized to total 3T6 DNA, digested with Bam HI or Eco R1. Out of 20 randomly chosen spcDNA clones, of which only two hybridized with any of the highly repeated probes (one for B1, one for R), all produced the hybridization smear characteristic of interspersed repetitive sequences. In some cases, bands of discrete sizes were visible over the smear. This predominance of dispersed repetitive sequences was unexpected since repetitive, non-satellite DNA makes out only 16 - 24 % of the mouse genome (24). Of this, the five previously identified highly repeated sequence families constitute 8 % of total DNA (24), leaving 8 - 16 % for middle repetitive sequences. Since a similar proportion (9 %) of highly repeated DNA is found in our spcDNA libraries, on the order of 90 % of our spcDNA clones represent dispersed, middle repetitive sequences. By comparing hybridizations where the inserts are of similar length, and where labeling of probes has been equally efficient (based on the hybridization to a standardized amount of plasmid DNA) we conclude that the majority of our interspersed spcDNA clones hybridize less intensely with 3T6 DNA than do the clones of highly repetitive DNA.

Representation of individual sequences in spcDNA from untreated and cycloheximide-treated cells. By probing duplicate filters with colonies from the clone banks with either total chromosomal 3T6 DNA or total 3T6 cccDNA, clones were identified that reacted somewhat stronger with cccDNA than with chromosomal DNA, which would suggest an overrepresentation in spcDNA. In this group, no clones hybridized with any of the highly repeated probes. Twelve such clones were used as probes against Southern blots of chromosomal DNA (Fig. 2, lanes 6 - 10). Just as with the randomly chosen clones, all showed a dispersed pattern of hybridization with one exception, pSPC-20, which labeled a set of distinct bands, repeated on the order of 10 - 100 times in the genome (Fig. 2, lane 8).

In Fig. 3, individual clones of highly repeated DNA have been

hybridized to CsCl/EtBr gradients of partially purified cccDNA from untreated or cycloheximide-treated cells. Approximately 90 % of the ^{3}H-activity was found in the linear peak in these gradients. The position of the circular peak can be verified by hybridizing to mtDNA (Fig. 3, rows 1 and 6). Hybridization by probes other than mtDNA to the circular peak consistently occured at a slightly higher position in the gradient. This is in agreement with the original observation of Smith and Vinograd (29), who attributed it to a higher superhelical density in spcDNA than in mtDNA. Of course, it is essential to this type of assay that no homologies exist between mtDNA and the probes used. This was verified by comparing the sequences of mtDNA and of the highly repeated probes (the sequenced parts in the case of MIF) using the GENEUS (32) Dot Matrix program.

The probe consistently yielding the most distinct circular peak is B1 (Fig. 3 A and B, rows 2 and 7). This peak is found both in untreated and cycloheximide-treated cells, and the increase upon cycloheximide treatment is only moderate (about two-fold in this assay). All other highly repeated probes (B2, EC, R, MIF and satellite) give comparable results with very little material hybridizing below the linear peak in untreated cells (Fig. 3 A, rows 3 - 5, and data not shown). However, after cycloheximide treatment a massive increase of hybridizing circular DNA is seen with these probes (Fig. 3 B, rows 8 - 10). This amplification is particularly prominent (five- to tenfold in this assay) with MIF and R, which in most cases are found adjacent in the chromosome (33).

DISCUSSION

When constructing clone libraries of spcDNA, there is a conflict between two major interests:

i) to clone intact circles, and

ii) to obtain a library that is unbiased with respect to sequence. The strategy in this work has been to use restriction enzymes that cleave DNA frequently (four-cutters) or combinations of several enzymes (six-cutters) in order to cut within an as wide as possible variety of sequences. This goal has been achieved since the majority of spcDNA has been

linearized. There is thus reason to believe that our spcDNA libraries represent a large fraction of the total sequence complexity in the spcDNA population.

A comparatively large number of spcDNA clones have been examined for the presence of the six most highly repeated sequence elements, permitting an evaluation of the distribution between highly repeated elements. It appears that the B1 repeat is overrepresented in spcDNA in 3T6 cells relative to the rest of highly repeated families, both from the number of positive clones in the libraries and from hybridizations to CsCl-EtBr gradients. In this context it is interesting to note that there exist sequence homologies between B1 and the papovavirus origin of replication (34). Ariga (35) demonstrated autonomous replication of a cloned Alu element, considered to correspond to B1 in primates, in extracts from CosI cells. This does not necessarily imply that Alu- or B1- containing spcDNA molecules replicate as episomes in vivo, although structures resembling replicative intermediates of circular molecules have been identified in the electron microscope by us and others (36). Rather, B1-containing circular molecules can have arisen as a result of "misfiring" from chromosomal origins of replication (6), since labeling data point at excision from chromosomes as the main mechanism of spcDNA formation (29).

In the only previously published cloning study of spcDNA from mouse cells, Fujimoto et al. (5) argue for the R repeat to be preferentially involved in spcDNA formation in mouse thymocytes. This may be a consequence of the specific genomic rearrangements in thymocytes since the immunoglobulin genes are associated with R repeats. We find no apparent deviation from the expected amount of R repeats in mouse 3T6 spcDNA. Jones and Potter (11) screened for sequences overrepresented in cycloheximide-amplified HeLa spcDNA and found sequences representing a minor, tandemly repeated, component of satellite DNA. Several explanations can be given for this result, apparently in conflict with those of us and others (37). First, the reason may reside in the differences in cell type or subcellular location of spcDNA (Jones and Potter isolated spcDNA from the cytoplasm of HeLa cells). Second, their use of cold competitor Alu DNA in

differential hybridization precluded any conclusions on the representation of Alu in spcDNA.

Overabundance of repetitive, mainly interspersed, vs. unique sequences in spcDNA has been found by us and others (2-4, this paper). The circular form of dispersed repetitive elements may be an intermediate in the process of their dispersal in the genome. When analyzing clones with long inserts, the presence of a highly repeated sequence element overshadows middle repeated and unique sequences. By virtue of the short mean length of our cloned inserts, we have been able to sort out that highly repeated DNA, as a whole, is not overrepresented in spcDNA. It seems as if what is really enriched in the spcDNA population is middle repetitive DNA, as a group, while unique sequences are not found to any great extent in spcDNA.

Our investigations of the amount of a certain sequence in circular form in untreated and cycloheximide-treated cells indicate that the sequence composition in the amplified spcDNA may differ from the normal counterpart. This question has not been directly adressed earlier. Some sequences, such as B1, seem to exist "constitutively" in circular form, whereas circularization of others, such as MIF and R, appears to be more strongly induced by cycloheximide.

The main aim of this work was to identify clones that would serve as efficient probes for spcDNA to study the process of its amplification. Candidate clones for this task are B1, by virtue of its high repetition degree and overrepresentation in spcDNA, and the MIF/R group by virtue of its apparent high degree of amplification upon cycloheximide treatment. Of the middle repetitive group, some clones may be suitable, although their lower repetition degree entails a lower sensitivity. It remains unknown whether the same or other sequences are amplified on cycloheximide treatment as after other stimuli that provoke an increase in spcDNA content.

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