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Characterization of repetitive sequence families in mouse heart small polydisperse circular DNAs: age-related studies

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ABSTRACT

Using alkaline denaturation-renaturation, exonuclease III digestion and density gradient centrifugations, we have isolated covalently closed circular DNA (cccDNA) molecules from 1-, 8-, 16-, and 24-month C57BL/6 mouse heart tissues. Electron microscopic analyses demonstrated that all these preparations contained small polydisperse circular DNAs (spcDNAs). spcDNAs showed similar size distributions at all ages, but more discrete size classes and slightly larger circles were observed in the 24-month heart spcDNA preparations. Based upon the final yields of spcDNAs, there appeared to be no age-related changes in the quantity of these circular molecules in vivo. Furthermore, [^{3}H]-pBR322 recovery studies revealed no endogenous factors that might have affected the yield of spcDNAs from young and old tissues. To determine if there were any age-related changes in the quantity of repetitive sequences in spcDNAs, we probed heart spcDNAs with B1, B2, IAP, L1 and satellite sequences of the mouse genome. The hybridization results showed that these sequence families were differentially represented at all ages in spcDNAs. B2 sequences were the highest across all the age groups while L1 sequences were the lowest. The quantity of B1-, B2-, IAP-, and L1-spcDNAs appeared to decrease at 24-months. Satellite sequences appeared to decrease from 1-month to 8-months, but no change beyond 8-months.

INTRODUCTION

Small polydisperse circular (spc) DNA molecules appear to be present in all eucaryotic cells. These molecules are extrachromosomal and can be physically separated from high molecular weight chromosomal DNA. Their size distribution, in general, is very heterogeneous, with measurements ranging from 150 base pairs (bp) to over 20,000 bp (1). The amount of these circular molecules varies from cell type to cell type. Reports of spcDNA content range from .001 to 0.1% of the total DNA (2). The size heterogeneity and low copy number of spcDNAs make isolation, cloning and characterization difficult by conventional techniques. However, spcDNAs have been recovered from HeLa cells (1,3), Drosophila cultured cells (4), Chinese hamster ovary cells (5), African Green monkey kidney cells (6), mouse tissue culture cells (7), and diploid human fibroblasts (8). Despite their heterogeneity, some cells in culture exhibit discrete size classes of spcDNAs in agarose gels (4).

One distinguishing feature of eucaryotic extrachromosomal DNAs is that they appear to be composed entirely of chromosomal sequences. For example, denaturation-renaturation studies have revealed that spcDNAs contain different sequence complexity classes, mirroring the organization of sequences found in genomic DNA (2-9). In addition, pulse-chase labeling experiments suggested that the circles might be derived from chromosomal DNA and the buoyant density of spcDNAs was shown to be very close to that reported for chromosomal DNA (1). Different sequence families that have been reported to be present in spcDNAs include short interspersed repetitive sequences (SINEs) such as Alu sequences of primates and the B1 and B2 sequences of rodents, long interspersed repetitive sequences (LINEs) such as L1, and tandemly repetitive sequences such as satellites (2-10). A smaller fraction of spcDNAs has a significantly higher sequence complexity, implying the presence of unique or low copy sequences (11).

Mouse B1 and B2 sequences contain RNA polymerase III promoters, have long poly-dA tracts at their 3' ends, are flanked by short direct repeats, and are abundantly transcribed into nuclear RNA (12). These features have led investigators to propose that these short sequences are transposable elements that move from one area of the genome to another through an RNA intermediate that is reverse transcribed to yield linear or circular double-stranded molecules (13). L1 sequences are transcribed by RNA polymerase II and are also thought to be reverse transcribed and subsequently transposed to a different region of the genome through an RNA intermediate (14). Intracisternal A-particles (IAPs) are retroviral-like elements that are interspersed and repeated approximately 1000 times in the mouse genome (15). The IAPs have been identified extrachromosomally as nucleoprotein particles. Like classical retroviruses, IAPs appear to code for their own reverse transcriptase and to transpose through an RNA intermediate (16). Unlike retroviruses, they do not go through an extracellular phase. In contrast to the above mentioned sequences, satellite (SAT) sequences are tandemly repeated, are not transcribed and are thought to be amplified by a saltatory replication mechanism or by unequal recombination (17).

Most of the reports characterizing and sequencing spcDNAs have been carried out on cultured cell lines. We view it likely that important cellular processes that occur in vivo also include spcDNA formation, expansion, and contraction. Indeed, extrachromosomal DNA amplification (18) and circular immunoglobulin gene recombination products (19,10) are two examples of extrachromosomal DNA molecules arising from developmental processes. To explore in vivo changes that might play a role in establishing and maintaining

differentiated cell function, we have isolated and characterized spcDNAs from several mouse tissues at different ages prior to initiating more detailed cloning and sequencing studies.

We report here that mouse heart tissues contain spcDNA molecules from 1 month to 24 months of age. The heart spcDNAs contained SAT and the moderately repetitive B1, B2, L1, and IAP sequences in various proportions that do not necessarily reflect their chromosomal copy number. The abundance of the repetitive sequence elements studied and the amounts and size distributions of spcDNAs stayed relatively constant throughout much of the lifespan of the mice. However, there seemed to be a decrease in dispersed repetitive sequences in spcDNAs and a slight increase in circle size in senescent mouse heart.

MATERIALS AND METHODS

Animals and tissues

The animals used in this investigation were the highly inbred strain of mice, C57BL/6. 1-month and 8-month old mice were purchased from Charles River Breeding Laboratories. 16-month and 24-month old mice were supplied from the colony maintained by the National Institute on Aging, where they were Caesarean-originated and maintained behind a barrier to exclude microbial pathogens. Mice were sacrificed and heart tissues were removed and immediately frozen and kept at -70°C until later use. The number of tissues per preparation varied from 10 to 20.

Isolation of spcDNAs

The procedure used was a modification of the alkaline extraction procedure of Birnboim and Doly (20). Heart tissues from 10 to 20 animals were minced in a homogenization buffer consisting of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0) and 5 mM $MgCl_2$ at 5 ml/g tissue. The tissues were disrupted at 0°C with a motor-driven teflon-pestle, glass homogenizer followed by 10 passes with a Dounce homogenizer. After incubation for 5 min on ice, two volumes of a 0.2 N NaOH, 1% SDS solution were added. The homogenate was kept on ice for 30 min, and then 0.5 volume of 3 M sodium acetate (pH 4.8) wwas added and gently mixed by inversion. The mixture was incubated on ice for one hr, and cleared by centrifugation at 25,000 xg for 20 min at 0°C. The supernatant was removed and recentrifuged as described above. The supernatant was collected and nucleic acids precipitated with an equal volume of isopropanol, kept at -70°C for 30 min, sedimented at 25,000 xg for 20 min at 0°C and dried *in vacuo*. The pellet was resuspended in 4 ml of TE buffer (0.01 M Tris-HCl, pH 8.0; 1 mM EDTA) and extracted with an equal volume of TE buffer-saturated

phenol:chloroform (1:1) and re-extracted until the interphase became clear. After isopropanol precipitation of the nucleic acids in the aqueous layer, the pellet was dissolved in 5 ml of exonuclease III buffer (66 mM Tris-HCl, pH 8.0; 1 mM DTT; 0.66 mM $MgCl_2$) and exonuclease III enzyme (Pharmacia) was added to a final concentration of 22 U/ul. After incubation at 37°C for 1 hr, RNAase A was added to a final concentration of 50 ug/ml and incubated at 37°C for 30 min. Proteinase K was then added to a final concentration of 100 ug/ml and incubated at 37°C for 1 hr. The digest was extracted four times with buffer-saturated phenol:chloroform, and the DNA precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol. The pellet was washed, dried *in vacuo*, resuspended in TE buffer and subjected to two sequential equilibrium sedimentations in ethidium bromide/cesium chloride gradients as described (21). Form I material (covalently closed circular, cccDNA) was collected and dialyzed against 0.1x TE buffer after removal of the ethidium bromide by 1-butanol extraction. The DNA was concentrated in a Speed-Vac (Savant) concentrator and resuspended in 2 ml of water.

Slot-blots and hybridizations

Following each equilibrium centrifugation step, fractions were collected from the bottom and 25 ul aliquots were slot-blotted as described by Kafatos, et al. (22). The filters were hybridized to nick translated DNA as described by Rigby, et al. (23). The fractions corresponding to the peak of hybridization associated with spcDNA molecules (1.6-1.63 g/ml, as determined by refractometry) were pooled, recentrifuged and concentrated as delineated above.

After quantitation of the DNA in each preparation, 50, 100, and 188 ul volumes were slot-blotted onto quintuplet nitrocellulose filters. Each filter was also slot-blotted with 1, 2, 4, 5, 10, 15, 20, and 30 ul volumes of a 50 ng/ml pBR322 preparation as an internal standard. The DNA was then hybridized to one of the following nick-translated repetitive sequences: B1, B2, IAP, L1, SAT. Pre-hybridization was performed overnight at 42°C in a solution containing 50% formamide, 6x SSC (0.9 M NaCl, 0.45 M sodium citrate), 0.5% SDS, 5x Denhardt's solution, and 100 ug/ml denatured salmon sperm DNA. After pre-hybridization, the denatured probes (5 x 10^7 input cpm) were added to the pre-hybridization solutions. Following a 24 hr hybridization at 42°C, the filters were washed 3 times at room temperature for 15 min each in 1x SSC, 0.1% SDS. Another wash was performed in 1x SSC, 0.1% SDS for 1 hr at 65°C. Filters were dried and autoradiographed for 4, 18, and 48 hrs.

Probes

The recombinant plasmids used as probes were pMR225 (B1), pMR142 (B2),

pMR134 (L1) (24), A-81 (IAP) (25), and pSAT (satellite) (26). The plasmid vector sequences of recombinants pMR134, A-81 and pSAT were pBR322. The vector sequences of recombinants pMR225 and pMR142 were pSP64, a derivative of the pUC12 plasmid carrying the SP6 polymerase promoter from Salmonella. The vector pSP64 shares approximately 70% homology with pBR322 (27). Insert sizes in all the recombinants except A-81 ranged from 100 to 250 base pairs. The insert size of recombinant A-81 was 6.8 Kb. The repetitive sequence probes B1, B2, and L1 did not hybridize to mitochondrial DNA (data not shown). IAP and satellite sequences were not tested for homology to mitochondrial DNA. Control hybridizations using pBR322 as a probe showed no homology between the plasmid sequences and the spcDNA preparations.

Estimation of yield as a function of age.

Plasmid pBR322 was labeled with [3H]-thymidine to a specific activity of 6.3 x 10^4 cpm/ug by chloramphenicol amplification (28). The percentage of Form I material in the purified [^{3}H]-pBR322 preparation was determined to be 80%. Plasmid (10^7 cpm) was added to 1 month and 24 month heart tissues prior to homogenization. Aliquots were removed at each step of the isolation procedure outlined above and radioactivity determined by scintillation counting. The values were adjusted for the amount of Form I material in the [^{3}H]-pBR322 preparation.

Determination of spcDNA mass

The mass of spcDNA was determined by either ethidium bromide fluorescence as described (29) or by diphenylamine assays (30).

Quantitation of repetitive sequences

Quantitation of repetitive sequences in spcDNAs of all the ages studied was done by densitometric scanning of the slot-blot autoradiographs. X-Ray films were scanned with a densitometer (LKB Ultra-Scan). The area of the peaks was determined by computer-assisted integration. Several exposures were obtained to ensure that area values used for calculations fell within the linear film range. Area units representing the increasing volumes of the heart spcDNA preparations were calculated for each of the probes used. The internal standard (pBR322) area unit values were calculated for each filter and plotted as a function of the volume. Linear regression analyses were used to determine the correlation coefficients and slopes of the lines for each probe. The insert size of the IAP probe was 6.8 kbp, representing 60% of the total amount of bases of the recombinant plasmid. Therefore, only 40% of the recombinant was pBR322 sequences. The slope values associated with hybridization of the IAP probe to the pBR322 internal standard were divided by a factor of 0.6. This calculation yielded a corrected slope for the binding of recombinant IAP vector sequences to

the pBR322 internal standard. The highest slope value was divided by each of the lower slope values to determine a correction factor. This factor was used to correct binding data for differences in specific activities between labeled probes in one experiment. The corrected total area units for each probe were divided by the total mass of spcDNAs recovered at each age.

Electron microscopy

After nicking with ultra-violet light in the presence of ethidium bromide (30 ug/ml), purified heart circular DNA molecules were visualized with an aqueous droplet technique as described earlier (31). The molecules were digitized and their length calculated using mouse mitochondrial DNA, (16.3 Kb) as a length standard (32).

RESULTS

In order to determine if the mass yield of spcDNA molecules varied as a function of age, or from experiment to experiment, three independent preparations per age group were performed. The results are shown in Table 1. The number of tissues varied for each preparation and/or age group. The results are expressed as ug of spcDNA per heart for each experiment. The results suggest that there is no age-related change in the mass quantity of heart spcDNAs. The range in values within one age group was as great or greater than between different age groups, e.g. 0.36-0.58 for 24-months. To further explore the possibility that there might be differential yields of covalently closed circular molecules between young and old tissues, we included a known amount of [^{3}H]-pBR322 in the buffer prior to homogenization of 1-month and 24-month hearts, and then calculated the recovery of cccDNA molecules. The results are shown in Table 2. Essentially identical recoveries of cccDNAs were obtained for 1-month and 24-month heart preparations. The biggest loss (close to 50%) occurred at the ethidium bromide/cesium chloride equilibrium density centrifugation step. After dialysis the percent yields of cccDNA molecules for 1-month and 24-month heart were 33% and 32%, respectively. It should be noted that no RNase treatment was included in this experiment, whereas the spcDNA isolation procedure did have an RNase step. Williams et al. (33) reported that chloramphenicol-amplified plasmids fail to remove all the RNA primers used in replication. This renders covalently closed circular plasmid molecules sensitive to RNase treatment. Thus, they become open circular and are lost during equilibrium centrifugations. Indeed, we confirmed these observations in a control experiment performed using [^{3}H]-pBR322 with or without RNase treatment (data not shown).

Table 1. Yields of purified cccDNAs from mouse heart tissues of different ages

Age (months)	Experiment Number	Number of Tissues per Preparation	Total ug cccDNAs	ug cccDNA/ Heart	Mean ug cccDNA/ Heart ± S.D.
1	1	10	3.30	0.33	
	2	15	6.60	0.44	
	3	15	5.04	0.34	0.37 ± 0.06
8	1	10	3.60	0.36	
	2	15	7.80	0.52	
	3	20	8.88	0.44	0.44 ± 0.08
16	1	10	4.50	0.45	
	2	15	7.80	0.52	
	3	20	10.32	0.52	0.50 ± 0.04
24	1	10	3.60	0.36	
	2	15	5.25	0.35	
	3	12	6.96	0.58	0.43 ± 0.13

In order to determine the location of spcDNAs in ethidium bromide/cesium chloride gradients, fractions were collected from the bottom and aliquots from each fraction were slot-blotted onto nitrocellulose filters and hybridized with a nick-translated repetitive sequence probe. After autoradiography, the blots were scanned with a densitometer. Two regions of hybridization were observed. The peak of material hybridizing at the higher density of 1.60 to 1.63 g/ml (fractions 13-19), as determined by refractometry, corresponded to spcDNA molecules (1). The peak of material hybridizing at the lower density of 1.57 g/ml (fractions 22-30) corresponded to open circular or linear DNA. Form I material was pooled and rebanded. Rebanding of the spcDNAs in a second ethidium bromide/cesium chloride gradient with subsequent hybridization to a mixture of B1, B2 and L1 radiolabeled probes showed that 91% of the radioactivity was found in the covalently closed circular DNA region of the gradient. The 9% of material found in the open circular/linear DNA region of the gradient was shown to be resistant to exonuclease III treatment at enzyme concentrations selected for preferential degradation of linear molecules (data not shown). Therefore, these resistant molecules were probably relaxed circles rather than contaminating linear molecules.

spcDNA molecules isolated from whole heart tissues consistently banded at a higher density than mitochondrial DNA molecules (data not shown), in agreement with Smith and Vinograd's report of a higher superhelical density for spcDNAs

Table 2. Recovery of covalently closed circular DNA molecules from young and old hearts.

Purification* Step	CPM ± S.D. (x10^6)#		Percent Recovery	
	1-month	24-month	1 month	24 month
Homogenate	10.0	10.0	100	100
Alkaline Denaturation/ Renaturation	9.1 ± 0.5	9.2 ± 0.4	91	92
Phenol:chloroform Extractions	6.3 ± 1.0	6.4 ± 0.4	63	64
Exonuclease III Proteinase K Digestions; Phenol:chloroform Extractions	6.0 ± 0.1	5.7 ± 0.1	60	57
CsCl/EtBr	3.3 ± 0.2	3.2 ± 0.5	33	32
Dialysis	3.3 ± 0.2	3.2 ± 0.2	33	32

*Purification was performed with 3 hearts in each age group. [^{3}H]-pBR322 was added to the preparation before homogenization.

#Mean ± S.D. of 5 independent volume determinations at each step of the purification procedure.

(1). This facilitated the separation of spcDNA molecules from mitochondrial DNA molecules. In fact, electron microscopic analyses confirmed the observation that mitochondrial DNA did not entirely copurify with the spcDNA molecules. The number of mitochondrial DNA molecules recovered as a function of age represented approximately 10% of the cccDNA preparations. In terms of mass, however, mitochondrial DNA accounted for approximately 50% of the total in all the ages studied. The mass yields shown in Table 1 therefore represent spcDNA and mitochondrial DNA molecules, both of which remained relatively constant with age. By incorporating an exonuclease III digestion of the partially purified DNAs, three consecutive rounds of density gradient centrifugations were not required. Control experiments using [^{3}H]-pBR322 and spcDNAs demonstrated that at the concentrations of enzyme used, linear molecules were preferentially digested while covalently closed circular molecules were left undisturbed (data not shown).

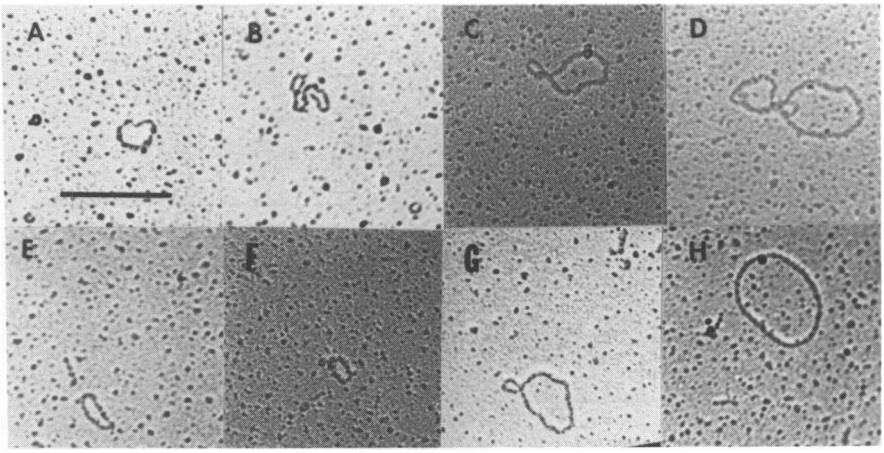

Figure 1. Electron micrographs of representative spcDNA molecules isolated from mouse heart tissues at different ages. Heart spcDNAs were isolated, purified and prepared for electron microscopy as described in Materials and Methods. The bar indicates 0.5 um. The circular DNA molecules shown have contour lengths, in um, of (A) 0.5, (B) 0.88, (C) 0.96, (D) 1.80, (E) 0.44, (F) 0.3, (G) 1.02, (H) 1.2. A and B, 1-month heart; C and D, 8-month heart, E and F, 16-month heart; G and H, 24-month heart. Magnification of all the micrographs was the same.

Several investigators have shown that the quantity and size distribution of spcDNA molecules change during development (19) and aging (34). In order to determine whether any age related changes could be observed in the size distribution of spcDNAs isolated from heart tissues, electron microscopic analyses of heart circles were performed. Figure 1 shows a composite electron micrograph of representative circular DNA molecules isolated from mouse heart tissues of different ages, and the size distributions are shown in Fig. 2. No spcDNA molecules larger than 10 Kb were observed. The size distributions were similar in all the ages studied. However, there was a tendency for molecules in old heart to be found in more discrete size classes (Fig. 2), particularly in larger spcDNAs (> 3.5 Kb, Table 3). In fact, the percentage of circles greater than 3.5 Kb was 28% and 39% in two independent 24-month heart spcDNA preparations whereas less than 20% of spcDNAs were greater than 3.5 Kb in the preparations from younger animals.

To determine if there was an age-related change in the relative abundance of repetitive sequences in spcDNAs, purified 1-month, 8-month, 16-month and 24-month heart spcDNAs were slot-blotted onto quintuplet nitrocellulose filters, and hybridized to nick-translated B1, B2, IAP, L1 or SAT sequences of the mouse genome. Each filter contained 8 increasing concentrations of a pBR322 DNA

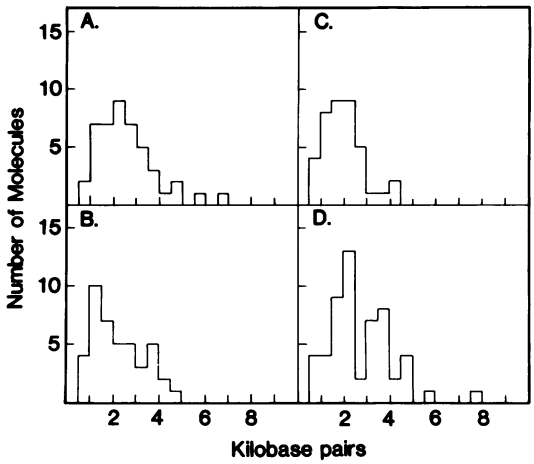

Figure 2. Size distribution of spcDNAs isolated from mouse heart tissues of different ages. Form I molecules were nicked and prepared for electron microscopy as described in Materials and Methods. (A) 1-month; (B) 8-month; (C) 16-month; (D) 24-month.

used as an internal standard. After hybridization, the autoradiographs were scanned to quantitate the degree of hybridization. Exposure of the film was shown to be linear up to 48 hrs. We chose to use an 18 hr exposure time for our analyses. Figure 3 is a composite autoradiogram showing the binding of all 5 probes to increasing volumes of heart spcDNAs from four different ages. Figure 3-A shows the binding of each probe to the experimental samples. Figure 3-B shows the binding of the probes to the pBR322 internal standard. The hybridization intensities of the standards were very similar, suggesting very little variability within each experiment in the hybridization efficiency of the probes. However, differences were observed when the heart spcDNAs were compared. B2 was the highest in hybridization intensity across the ages. For a single probe, differences between the ages partly reflect differences in total spcDNA yields. Differences between probes for a single age are differences in the content of sequences in spcDNAs. The area units associated with the internal standard were plotted as a function of the volume of the pBR322 DNA applied to the filters. A linear relationship was observed for both the pBR322 internal standard and the experimental samples. This is demonstrated in Figure 4 which shows a representative example using B1 and B2 repetitive sequences as probes. Figure 4-A shows that binding of B1 and B2 probes to increasing concentrations of 16-month heart spcDNAs is linear. There is approximately a 1.5-fold difference

Table 3. Size distribution of small circular DNAs from mouse heart cells at different ages

Age	Number of Molecules Measured	Number-Average Size ± S.D. (Kb)	Fraction of Circles > 3.5 Kb
1 month	45	2.8 ± 1.6	0.18
8 month	42	2.5 ± 1.1	0.19
16 month	39	2.3 ± 1.5	0.08
24 month*	53	3.0 ± 2.1	0.28
24 month*	36	4.1 ± 3.2	0.39

* Different preparations of 24-month heart spcDNAs.

between B2-homologous spcDNAs and B1-homologous spcDNAs. Figure 4-B shows the resultant hybridization of probe plasmid sequences to the internal standard. The lines generated with L1, SAT, and IAP probes showed the same patterns. The lines for all 5 probes had correlation coefficients of ≥ 0.97.

It was important that all probes be bound to the filters with the same efficiency, in order to make comparisons between samples. To correct for slight differences in hybridization within each experiment, the slopes of the lines of

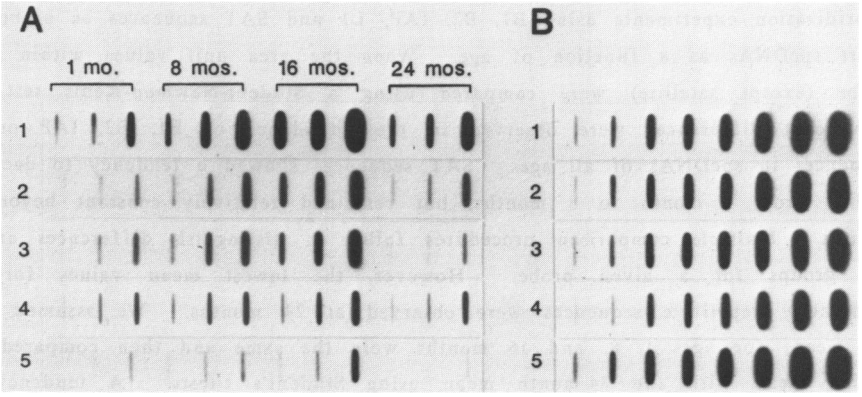

Figure 3. Hybridization of repetitive sequences. (A) Autoradiograph showing hybridization of repetitive sequences to increasing concentrations of 1-month, 8-month, 16-month and 24-month heart spcDNAs. (B) Hybridization of vector sequences to increasing concentrations of pBR322. The probes used in A and B were: B2 (1), B1 (2), IAP (3), SAT (4), and L1 (5).

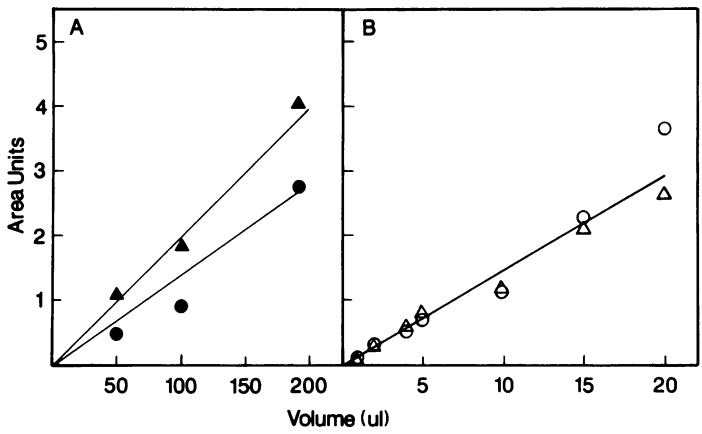

Figure 4. Analysis of efficiency of binding of B1 and B2 probes. Heart spcDNAs were isolated, slot-blotted, hybridized, autoradiographed and scanned as described in Materials and Methods. Increasing concentrations of pBR322 DNA (50 ng/ml) were included in the filters as an internal standard. (A) 16 month heart, ●—● B1; ▲—▲B2; (B) pBR322, ○—○B1; △—△B2.

the internal standard were used to normalize all the experimental sample values. In addition, since the total micrograms of spcDNAs recovered varied according to the number of tissues used (Table I), the total area units were divided by the total micrograms of spcDNAs recovered in each preparation. A summary of the hybridization experiments is shown in Table 4. This table represents 5 hybridization experiments using B1, B2, IAP, L1 and SAT sequences as probes of heart spcDNAs as a function of age. When the area unit values within each probe (except satellite) were compared using a Student-Newman-Keuls test, no significant differences were observed in the abundance of B1, B2, IAP or L1 sequences in spcDNAs of all ages. SAT sequences showed a tendency to decrease ($p<0.1$) from 1 month to 8 months, but remained relatively constant beyond 8 months. Multiple comparison procedures failed to distinguish differences among age groups for a given probe. However, the lowest mean values for all moderately repetitive sequences were observed at 24 months. We assumed that the means for ages 1, 8, and 16 months were the same and then compared the pooled mean with the 24-month mean using Student's t-tests. A tendency to decrease for B1, B2 and IAP was observed at 24 months. However, this decrease was not statistically significant ($p < 0.1$). A significant ($p < 0.02$) decrease at 24 months was observed for L1 sequences. No significant age-related changes were observed with satellite sequences when the same analysis was used. Table 4

Table 4. Abundance of repetitive sequences in heart spcDNAs as a function of age.

Age (months)	Repetitive Sequence Probe*				
	B1	B2	IAP	L1	SAT
1	2.9 ± 1.7	4.2 ± 3.0	1.6 ± 0.4	0.7 ± 0.3	1.6 ± 0.5
8	2.2 ± 1.1	4.2 ± 1.8	1.3 ± 1.1	0.9 ± 0.3	0.8 ± 0.1
16	2.7 ± 1.2	4.7 ± 3.7	2.1 ± 2.5	1.2 ± 0.5	1.0 ± 0.3
24	1.7 ± 0.8	2.0 ± 1.5	1.0 ± 0.2	0.5 ± 0.3	0.8 ± 0.3

* Data are expressed as units of probe bound per microgram of spcDNA determined from microdensitometric scans of autoradiograms ± S.E. The values are the averages of five hybridization experiments with three independent spcDNA preparations.

also shows that B2 sequences are the most abundant in mouse heart spcDNAs across all the ages studied, while L1 sequences are the lowest.

Figure 5 shows the pooled means ± S.E. for each probe up to 16 months of age. A Student-Newman-Keuls test showed that B2 sequence-containing spcDNAs were greater than all the other sequences. B1 sequence-containing spcDNAs were different from L1 and SAT, but not statistically different from IAP. No differences were found between L1-, IAP- and SAT-spcDNAs. These data confirm previous reports that repetitive sequences of the mouse genome are differentially represented in extrachromosomal DNAs in vivo (35) and in vitro (7).

DISCUSSION

It has been suggested that the spcDNAs identified in eucaryotic cells represent the circular DNA intermediates generated during transposition or genomic rearrangements (36). We probed spcDNAs isolated from hearts at four different ages with B1, B2, IAP, L1, and SAT sequences of the mouse genome. We found the quantity of these sequences to be different in spcDNAs. In Table 5 the spcDNA hybridization results are expressed as a ratio of L1 sequences (the lowest representative sequence in this study). The spcDNA representation ratio was compared to the same ratio of genomic sequences. The abundance of B1, B2, and IAP repetitive sequences in spcDNAs was very different from their representation in the genome. Compared to their respective amounts in the chromosome, B2 was expanded extrachromosomally 33 times more than L1, B1 was

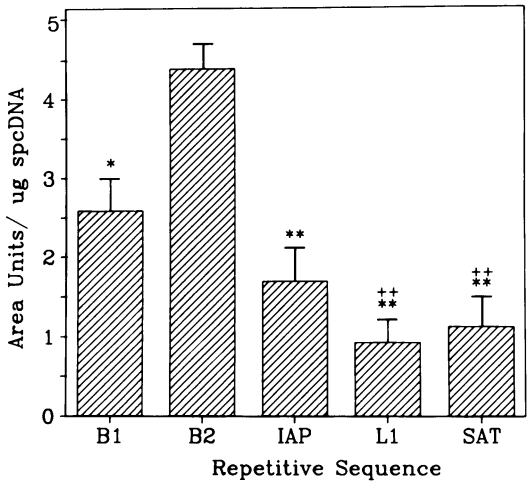

Figure 5. Relative abundance of repetitive sequences in heart spcDNAs. The bars represent the means ± S.E. of the combined values (from Table 4), for 1-, 8-, and 16-month age groups. * $p < 0.1$; ** $p < 0.05$; ++ $p < 0.01$.

expanded 12 times more than L1, and IAP 26 times more than L1. Sunnerhagen et al. (7) reported that B1 sequences were enriched in spcDNAs isolated from mouse 3T6 cells, while Fujimoto et al. (10) reported an over-representation of L1 sequences in spcDNAs isolated from mouse thymocytes. We believe these differences to be biological in nature, and that each cell type has a characteristic distribution of sequences in spcDNAs. In fact, we have detected differences between heart, liver, and brain tissues in spcDNA repetitive sequences (35, manuscript in preparation).

If B1, B2, IAP, and L1 are transcribed and then reverse transcribed to yield extrachromosomal DNA intermediates, it is possible that the spcDNAs detected in vivo are the results of such processes. Levels of B2-homologous spcDNAs might be higher than the levels of B1-homologous spcDNAs (in the genome, the copy number of B1 sequences is higher than the copy number of B2 sequences) because B2 sequences are more efficiently transcribed, as was proposed earlier (35). Satellite sequences are the only repetitive sequences used in this study that are not transcribed and thus should not arise by retrotransposition. When the levels of SAT sequences in spcDNAs were compared to B1, B2, IAP and L1 sequences, they showed a different pattern of representation: SAT sequences had the same abundance in spcDNAs as in the genome. Unequal recombination of these sequences followed by excision could give rise to satellite-homologous

Table 5. Representation of repetitive sequences in mouse heart spcDNAs.

Repetitive Sequence	Fraction of the Genome (Ref.)	Repeat Element in spcDNAs / L1 in spcDNAs*	Repeat Element in Genome / L1 in Genome
B1	0.9 - 1.3% (25)#	2.9	0.24
B2	0.5 - 0.8% (25)	4.6	0.14
IAP	0.2 - 0.4% (26)	1.8	0.07
L1	4.0 - 5.0% (25)	1.0	1.0
SAT	8.0 - 10% (46)	1.4	1.8

* Mean Area Units/ ug for each probe for all the ages from Table 4.
# Mean range values were used for calculations.

spcDNAs. Some spcDNAs in Chinese hamster ovary cells (37) and HeLa cells (38) seem to be generated by this mechanism.

Hypotheses about aging (for a review see 39) assume major perturbations in biological systems when in reality the changes could be more subtle and almost undetectable. The age-related studies reported in this investigation certainly support this notion. Increased plasticity, instability and reorganization of the cellular genome have been proposed to explain the decreased functional ability of senescent cells (40). For example, Shmookler Reis and Goldstein (41) showed that a major human repeat sequence family decreases in the genome during serial passage of diploid fibroblasts. In Podospora, a fungus, senescence is thought to occur through excision and amplification of short sequences (sen-DNAs) from the mitochondrial genome. These sen-DNA sequences are rearranged in the mitochondrial genome of senescence-resistant mutants (42). We wanted to determine if there were any changes in the sequences, mass, and size distribution of spcDNAs isolated from in vivo mouse heart tissues as a function of age. Heart is an appropriate organ to study aging because it appears to have a stable cellular population. In this context, the DNA content of mouse heart cells has been reported to be constant throughout the lifespan of this strain (43). Quantitation of the mass of spcDNA in each preparation suggested that recovery of heart spcDNAs did not change with age (Table 1). Moreover, to ensure that no endogenous factors could account for preferential yields of spcDNA molecules in heart, studies using [^{3}H]-pBR322 as an internal standard were carried out.

There appeared to be no non-specific factors that preferentially trapped or degraded circular molecules in either young or old hearts (Table 2).

The distribution of sizes remained relatively constant, with most molecules in the 2.3-2.6 Kb range. However, there seemed to be a tendency for more discrete size classes to appear as larger circles at 24 months of age. (Fig. 3 and Table 3). Using a mica-press-adsorption electron microscopic technique, Yamagishi et al. (44) showed that a restricted size class of spcDNAs of approximately 1.5 Kb was amplified several-fold in peripheral blood lymphocytes of 26-month old senescence resistant mice. In senescence-prone mice, the amplification of the small circular DNAs was observed as early as 10-weeks. Our results suggest that there was neither an amplification nor a decrease in the total quantity of the circles as a function of age. However, particular sequences, when studied individually could show changes during development or aging. No age-related changes occurred in vivo in B1-, B2-, IAP-, and L1-spcDNAs up to 16-months of age. On the other hand, satellite sequences showed a tendency to decrease from 1-month to 8-months, and no change beyond 8-months (Table 4). Mouse heart tissues go through a developmental polyploidization process that lasts into the first week of post-natal life (45). At these stages, DNA is being replicated without cell division. During this period, the replication machinery of the cells is very active, and satellite sequences could be concomitantly expanded chromosomally and extrachromosomally.

At 24 months, a tendency to decrease was observed in B1-, B2-, L1- and IAP-spcDNAs. This decrease could be explained by increased turnover, decreased synthesis, or increased insertion into the genome. Because the mass of heart spcDNAs remained relatively constant with age (Table 1), a decrease in the quantity of moderately repetitive sequences in spcDNAs should be accompanied by the appearance of other sequences that are not homologous to the probes we used. At 24 months we observed the appearance of more discrete larger size classes of spcDNAs. We do not know if there is any relationship between these two events, but it would be of interest to extend these studies to mice older than 24 months and determine if the same inverse correlation is magnified in very old mice.

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