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ARTICLE

Human mtDNA sublimons resemble rearranged mitochondrial genoms found in pathological states

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Sublimons, originally identified in plant mitochondria, are defined as rearranged mtDNA molecules present at very low levels. We have analysed the primary structures of sublimons found in human cells and tissues and estimated their abundance. Each tissue of a given individual contains a wide range of different sublimons and the most abundant species differ between tissues in a substantially systematic manner. Sublimons are undetectable in ρ^0 cells, indicating that they are bona fide derivatives of mtDNA. They are most prominent in post-mitotic tissue subject to oxidative stress. Rearrangement break-points, often defined by short direct repeats, are scattered, but hotspot regions are clearly identifiable, notably near the end of the D-loop. The region between the replication origins is therefore frequently eliminated. One other hotspot region is located adjacent to a known site of protein binding, suggesting that recombination may be facilitated by protein–protein interactions. For a given primary rearrangement, both deleted and partially duplicated species can be detected. Although each sublimon is typically present at a low level, at most a few copies per cell, sublimon abundance in a given tissue can vary over three orders of magnitude between healthy individuals. Collectively, therefore, they can represent a non-negligible fraction of total mtDNA. Their structures are very similar to those of the rearranged molecules found in pathological states, such as adPEO and MNGIE; therefore, we propose that, as in plants, human mtDNA sublimons represent a pool of variant molecules that can become amplified under pathological conditions, thus contributing to cellular dysfunction.

INTRODUCTION

Rearranged mitochondrial DNA (mtDNA) molecules, including both heteroplasmic deletions and partial duplications, are found in a great variety of human pathological states, notably in syndromic disorders affecting heart and skeletal muscle, the central nervous system and other post-mitotic tissues (1–3). These disorders can be divided into two categories, based on inheritance patterns and the types of rearranged molecule present, although their clinical features overlap. The first category shows sporadic occurrence or occasionally maternal inheritance, with a single primary rearrangement found in the affected tissue(s). This is presumed to have expanded clonally during early development. The second category shows autosomal inheritance, with multiple rearranged molecules found in different tissues, although individual cells or muscle fibre segments may contain just one or a few clonally expanded, rearranged mtDNA species (4).

The precise rearrangements found in the two categories of disorder show some similarities and also some differences. Clonally expanded 'single' rearrangements are often present in different forms that are theoretically interconvertible by homologous recombination (5,6), as illustrated in Figure 1. Rearranged molecules with a given pair of primary break-points can be present within a single individual simultaneously as deletions, partial duplications, deletion dimers and other multimers. Such break-points frequently occur at short, directly repeated sequences of up to 13 bp, suggesting that illegitimate recombination is the mechanism of primary rearrangement, although replication slippage has also been proposed. The deletion usually lies within the region between the replication origins for the two strands, although the exact break-points are scattered. Different deletions can be associated with similar clinical phenotypes and vice versa. Rearranged molecules are invariably a substantial fraction of total mtDNA in affected tissues, such as brain and skeletal

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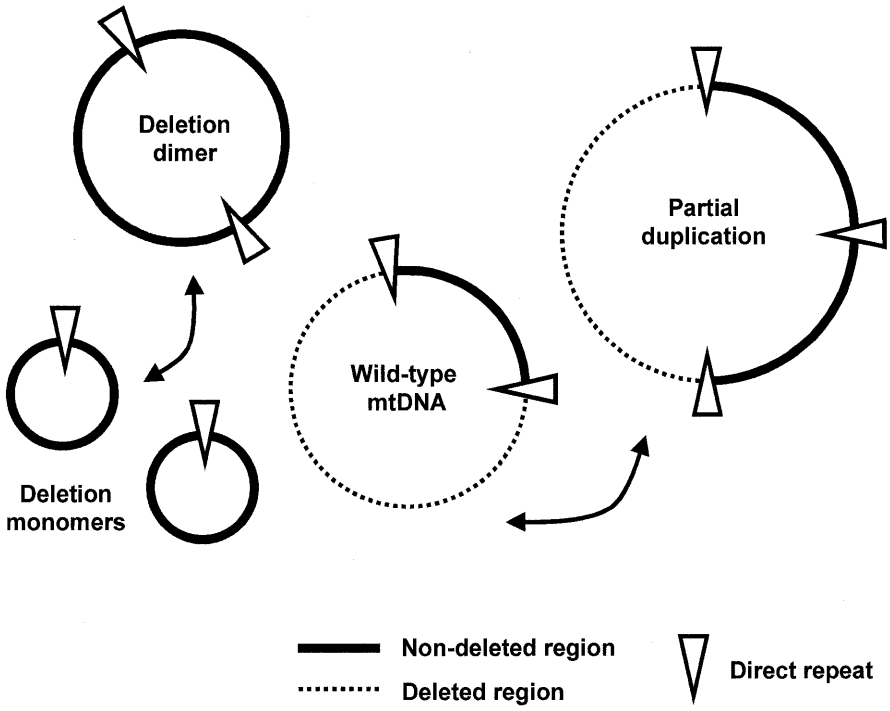

Figure 1. Interconversion of rearranged mtDNAs by homologous recombination. Different molecular forms as illustrated can carry an identical primary rearrangement, commonly arising from an illegitimate recombination event between two short direct repeats, shown as triangles. Homologous recombination between deleted and wild-type mtDNA creates a partial duplication and also triplications etc. Homologous recombination between deleted mtDNA molecules creates deletion dimers and higher order multimers.

muscle, in patients with Kearns–Sayre syndrome or sporadic ocular myopathy (PEO).

Multiple deletions, as found in such disorders as autosomal dominant PEO (adPEO) (7,8), mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (9,10) and inflammatory muscle disease, such as sporadic (11) and hereditary (12) inclusion body myositis (IBM), are also usually located between the replication origins, but break-points are more clustered. In particular, one end of the deletion very commonly lies within a short region near to the end of the D-loop. Direct repeats at break-points are rarer, or sometimes very short, and short direct and inverted repeats are often found near rather than at the exact break-points. Although each individual deleted species is present typically at a low level, deleted molecules as a whole again represent a substantial fraction of mtDNA (10–50% or more) in affected, post-mitotic tissues (10,13).

Recently, we as well as others have detected rearranged mtDNAs even in control subjects, using long PCR methods (14–16). Since such molecules cannot be detected by Southern blot, at least in young, healthy subjects, it has been assumed that they are present only at very low levels. At high template concentration, however, long PCR preferentially amplifies these molecules and exaggerates their apparent abundance. Reconstruction experiments that we performed showed that deleted molecules present at one or just a few copies per cell are easily detected by long PCR (17). Blot hybridization indicated that these molecules generally lack the region between the replication origins, just like the deleted mtDNAs found much more abundantly in cases of mitochondrial disease. Some reports, using PCR, have suggested that such deleted mtDNAs accumulate significantly during the course of ageing (18–22), as well as in some cases of dilated cardiomyopathy (23).

In an analogy with plant mitochondria (24,25), we have tentatively termed these low abundance rearranged mtDNAs 'sublimons' (14). The term was originally applied to low abundance DNA molecules found in the mitochondria of healthy (fertile) maize plants, whose structures were similar to those of rearranged mtDNAs found in certain male sterile lines (24). However, some very basic questions remain regarding the structure, abundance and tissue distribution of these molecules. In this paper, we present a detailed structural characterization of the sublimons present in heart muscle and compare the sublimon profiles of different individuals and tissues for clues as to the mechanisms that might generate them, in order to assess their relationship with pathological deletions.

The results confirm that heart sublimons have very similar structures to those of pathological multiple deletions. The same primary rearrangement can be represented as deleted or partially duplicated mtDNA, as well as deletion multimers. Break-points, frequently involving short direct repeats, are clustered near the end of the D-loop and in at least one other region of protein binding and the most abundant sublimon classes show break-point heterogeneity.

Remarkably, the spectrum of sublimons varies between tissues, but very similar profiles of sublimons are present in a given tissue from different individuals. This tissue specificity

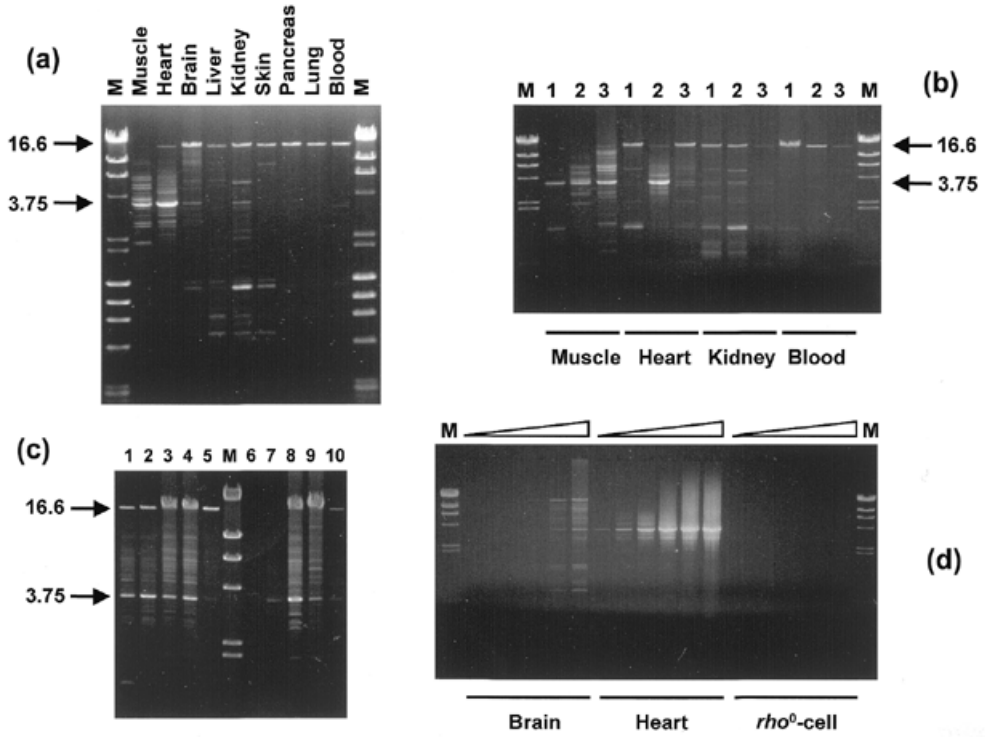

Figure 2. Detection of mtDNA sublimons by long PCR. All panels show agarose gel analyses of products synthesized using a primer pair located in the replication origin region. M, molecular weight marker [λ *Hin*dIII digest, combined in (a) with ΦX174 *Bsu*RI digest]. (**a**) Sublimon products obtained using template DNAs extracted from different tissues of a single individual (s2, 80-year-old male) post mortem, as follows: skeletal muscle (13 ng), heart (5.2 ng), brain (frontal cortex, 14 ng), liver (56 ng), kidney (13 ng), skin (41 ng), pancreas (12 ng), lung (57 ng) and blood leukocytes (38 ng). The 16.6 kb linear mtDNA product and the prominent 3.75 kb sublimon product obtained from skeletal muscle, heart, kidney and liver, are indicated by arrows. (**b**) Sublimon products obtained from four tissues of each of three individuals post mortem. Lanes 1, 2 and 3 used template DNAs from individuals s1 (22-year-old male), s2 and s3 (75-year-old female), respectively, as follows. Skeletal muscle: s1, 125 ng; s2, 13 ng; s3, 72 ng. Heart: s1, 24 ng; s2, 5.2 ng; s3, 2.0 ng. Kidney: s1, 26 ng; s2, 13 ng; s3, 18 ng. Blood: s1, 90 ng; s2, 38 ng; s3, 28 ng. Major products as in (a) are indicated by the arrows. (**c**) Sublimon products from DNAs extracted from heart muscle of five male alcoholic cardiomyopathy patients (lanes 6–10, ages 47, 65, 46, 46 and 48 years, respectively), plus five male controls (lanes 1–5, ages 65, 58, 52, 62 and 48 years, respectively). Approximately 10–100 ng of template DNA were used in each case. Major products as in (a) are indicated by the arrows. (**d**) Sublimon products from increasing amounts of template DNAs extracted from brain (0.02, 0.12, 0.58, 2.9, 14, 72 ng) and heart (0.21, 1.0, 5.2, 26, 130, 652 ng) of subject s2 post mortem, and from 143B osteosarcoma-derived ρ^0 cells (0.35, 1.8, 8.9, 44, 222, 1108 ng). A second (A549 lung carcinoma-derived) ρ^0 cell template (data not shown) also gave no products using the mtDNA primer pair. Both ρ^0 cell template DNA preparations gave efficient amplification of all single-copy nuclear DNA markers tested (data not shown).

applies even at the microscopic level, with different and characteristic patterns of break-point heterogeneity in each tissue. Based on a semi-quantitative multiplex PCR assay and counting a set of species exhibiting break-point heterogeneity within a limited region as a single sublimon, the more abundant sublimons may be present at up to 190 copies per cell, although in other individuals their representation can be much less than 1 copy per cell or even below the detection limit. We did not find any evidence of a simple relationship between age and sublimon abundance.

RESULTS

Sublimons are tissue specific

As previously (14,17), we used long PCR with adjacent, control region primers, to amplify full-length mtDNA, as well as any rearranged molecules containing deletions or partial duplications that were present in each DNA sample analysed. Note that the method, based on outwardly orientated primers, does not distinguish between deleted and partially duplicated molecules, which give identical products for a given primary rearrangement that still contains the priming sites. The pattern of products representing rearranged mtDNAs was different in the various tissues of a given individual (Fig. 2a). Products were in general shorter than 6 kb, although some longer products were also generated. A very prominent product of 3.75 kb was evident, for example, in both heart and skeletal muscle, but the same product was either much less abundant or not evident at all in other tissues. The other major products from heart and skeletal muscle were not identical, each tissue showing a set of products that were either unique or much less prominent in other tissues. The template concentration artefact, in which small circular molecules are preferentially amplified at high template DNA concentrations, makes it hard to judge accurately the relative amounts of sublimons in different tissues. However, based crudely on the amount of template dilution needed to give a substantial yield of the 16.6 kb wild-type mtDNA band, sublimons were markedly more abundant in post-mitotic tissues proposed to be subject to oxidative stress (heart, skeletal muscle, brain, as well as liver), but less abundant in other tissues such as skin, pancreas and blood. In

Table 1. Sublimons represented as cloned PCR products from control heart muscle

Clone	Size (bp)	Direct repeat (bp)	1st break-point (nucleotide positions)	2nd break-point (nucleotide positions)
6	940	6	633–639	16262–16268
11	1351	11	887–898	16105–16116
2	2170	0	1672	16071
8	2268	1	1770/1771	16071/16072
16	2397	1	1906/1907	16078/16079
10	2403	0	1906	16072
5, 15	2563	4	2064–2068	16070–16074
3, 13	2827	0	2333	16075
17	2830	1	2333/2334	16072/16073
4	3062	0	2540	16047
18	3279	0	2781	16071
14	3309	0	2818	16078
9	3753	1	3255/3256	16071/16072
21	3754	0	3255	16070
1	3759	1	3262/3263	16072/16073
19, 22	3760	2	3261–3263	16070–16072
23	3762	1	3262/3263	16069/16070
20[a]	3805	0	3270	16034
24	3807	0	3272	16034
5B[b]	3987	0	3485	16067
4B	4046	1	3548/3549	16071/16072
2B	4138	3	3308–3311	15738–15741
1B	4176	0	3575	15968
3B	4193	0	3692	16068
48	4237	6	3736–3742	16068–16074
43	4324	0	3826	16071
38[c]	4600	10	3568–3578	15537–15547
37	4637	2	4139–4141	16071–16073
33	4678	1	3548/3549	15439/15440
34, 36	4867	0	4376	16078
40	4953	4	4454–4458	16070–16074
45	4980	4	4482–4486	16071–16075
47	5693	0	5195	16071
42	5821	0	5328	16076
49	5822	2	5328–5330	16075–16077
44	6169	0	5671	16071
35[d]	6285	1	5786/5787	16071/16072
71	7760	0	7268	16077
92	8126	1	7628/7629	16071/16072
72	8138	7	7508–7515	15939–15946
90	8142	0	7644	16071
80	8164	1	7666/7667	16071/16072
70	8229	0	7732	16072
81	8309	4	7807–7811	16067–16071

Table 1. *Continued.*

Clone	Size (bp)	Direct repeat (bp)	1st break-point (nucleotide positions)	2nd break-point (nucleotide positions)
69	8316	6	7818–7824	16071–16077
89	8418	2	7919–7921	16070–16072
73	8527	5	8029–8034	16071–16076
79	8531	2	8031–8033	16069–16071
88[e]	8563	0	7399	15405
74	8591	0	8093	16071
91	8827	1	8291/8292	16033/16034
87	8875	1	8372/8373	16066/16067
82	9061	3	8561–8564	16069–16072
66–68, 83–85	9132	12	8636–8648	16073–16085
65[f]	9132	12	8636–8648	16073–16085
7[g]	n/a	n/a	n/a	

[a]T→C (nucleotide position 3264) near the break-point.
[b]1 bp insertion (T) at the break-point.
[c]Additional 8 bp direct repeat on opposite sides of the break-point.
[d]A→G (nucleotide position 16 074) near the break-point.
[e]7 bp direct repeat adjacent to the break-point.
[f]T→A (nucleotide position 8634) near the break-point.
[g]Mispriming artefact at nucleotide position 16 257.

contrast, very similar patterns of products were generated from template DNAs of a given tissue from different individuals: heart DNAs typically generated a 'heart-specific' set of products (Fig. 2c), although with some exceptions (Fig. 2b), whereas skeletal muscle or kidney DNAs gave more muscleor kidney-specific patterns (Fig. 2b). Repeated deproteinization of DNA samples by prolonged proteinase K digestion and phenol extraction made no difference to the pattern or abundance of products generated, indicating that they are not the result of residual protein contamination of the templates.

Sublimon-derived products were also amplified from cultured cell line DNAs (data not shown). However, such products could not be generated from either of two different ρ^0 cell templates (Fig. 2d), indicating that they are derived from bona fide mtDNA and are not the products of amplification from nuclear pseudogenes of mtDNA. Pre-digestion of the template with *Bam*HI, which cuts human mtDNA once, at nucleotide position 14 258 in the *ND6* gene, abolished synthesis of the 16.6 kb product, but almost all of the sublimon products were synthesized just as efficiently as from uncut DNA, indicating that the region in which the *Bam*HI site lies is deleted from almost all of the sublimons (data not shown). This applied to all tissues tested.

Sublimon break-points are concentrated in hotspot regions

To determine the primary structure of a representative set of sublimons, we cloned long PCR products from control heart DNA of a single individual, initially at random and subsequently by pre-selecting gel-purified products in the >4 kb size class, to counteract the inevitable bias towards cloning shorter products. Shorter cloned products were sequenced in their entirety, longer products as far as the break-points that defined the corresponding rearrangement. Most of the products characterized in the initial survey were equivalent to very large deletions, removing the previously assigned origin of light-strand synthesis O_L. The combination of long PCR and plasmid cloning discriminates against longer sublimons. However, some heart sublimons detected by long PCR were clearly in the size range >7 kb, so would retain O_L if their deletions began in or near the D-loop. To amplify sublimon-derived products whose deletions did not include O_L we used, as previously (14), a different pair of primers: one located in the D-loop, the other in the *COXI* gene. Nested PCR using these primers on gel-purified products of the first PCR reaction in the 7–15 kb size range gave an identical set of final products (data not shown). The findings from the full set of cloned products are summarized in Table 1 and Figure 3. Only one clone of 65 represented a mispriming artefact: the remainder were correctly primed products containing an internal mtDNA rearrangement.

The rearrangement break-points were concentrated in specific hotspot regions, the clearest example being a common break-point region (CBR) towards the end of the D-loop, around nucleotide position 16 070, which was involved in >80% (52 of 64) of the clones, although the exact break-point was heterogeneous (Fig. 3b). A less prevalent break-point region around nucleotide position 3260 was found in eight clones, in six of which the second break-point was within the CBR. The other two had a break-point at nucleotide position 16 034, which was also found in one other clone. The highly prominent long PCR product of 3.75 kb (Fig. 2) represents heart sublimons bounded on one side by the nucleotide position 3260 break-point region and on the other by the CBR. A second prevalent rearrangement, represented as seven clones,

was a 7.4 kb deletion, bounded on one side by the CBR and on the other, at nucleotide positions 8637–8648, by an exact 12 bp repeat of a sequence found at the CBR. All clones analysed represented molecules in which a large portion of the genome adjacent to the D-loop was deleted.

Several other features emerged from sequence analysis. In general, aside from these gross rearrangements, sublimon sequences matched those of bona fide mtDNA, apart from a small number of polymorphisms and probable PCR-induced mutations. The widespread scatter of low level substitutions and microdeletions characteristic of nuclear pseudogenes of mtDNA were absent. Less than half of all sublimons (28 of 64) showed evidence of significant direct repeats (>1 bp) at the break points (Fig. 3c), but the presence of short direct or inverted repeats very close to (but not exactly at) the rearrangement sites was commonly noted. Nine clones had direct repeats of 10 bp or more across the junctions, although seven of these represented the 7.4 kb deletion. One clone had a 1 bp insertion at the break-point. Note that because the primers used for PCR lie far from the rearrangement break-points, these sequence features are very unlikely to be PCR-induced artefacts and must instead represent the intrinsic structures of the sublimons themselves.

Microscale analysis reveals tissue specificity not individual specificity

The sequence analysis described above indicated microscale break-point heterogeneity even for 'classes' of sublimons that resolve as a single PCR product on agarose gels, e.g. the prevalent 3.75 kb species. To investigate this further, conventional PCR using one fluorescent and either of two non-fluorescent primers was used to amplify across the break-points of two of the major sublimon classes detected by sequencing: the prevalent (3.75 kb) sublimons, as well as a sublimon class of 2.83 kb, had one break-point also in the CBR, and the other within the 16S rRNA gene (represented as clones 3, 13 and 17). These primer pairs were tested first on myocardial DNA to confirm that they gave the predicted products (Fig. 4).

The products showed a similar heterogeneity as was evident from sequence analysis, although the patterns of heterogeneity were different for the two sublimon classes. The prevalent sublimon class manifested as 27 distinct products, differing in size by increments of one nucleotide, although these may represent many more than 27 different molecular species, since heterogeneity at both break-points was evident from sequence analysis. The second sublimon analysed, with a break-point in the 16S rRNA gene, manifested as four discrete products (shoulders on each of these peaks can probably be discounted, since they are attributable to the standard PCR artefact of incomplete addition of non-templated terminal A, by *Taq* DNA polymerase). Neither primer pair amplified any product from ρ^0 cell DNA, in a multiplex reaction in which a single copy nuclear gene control was efficiently synthesized. The products yielded by these primers are therefore specific to mtDNA.

Fluorescent PCR was used to analyse further the structure and representation of the prevalent 3.75 kb sublimon class in different tissues of the same individual, post mortem (Fig. 4a). This revealed a subtle but clear difference in the pattern of products derived from the various tissues. Skeletal muscle gave the least heterogeneous picture, with only two major products representing sublimons of 3762 and 3785 bp, plus a third product corresponding with a 3770 bp species. The patterns of products were highly reproducible for a given DNA sample. Even more remarkably, very similar profiles were produced using template DNAs from a given tissue of different individuals (Fig. 5). This was particularly striking in the case of heart muscle, where the product profiles from virtually all individuals, resolved at the nucleotide level, were almost exactly superimposable. In skeletal muscle samples, a product corresponding with a prevalent sublimon of 3762 bp was found in all individuals surveyed. The amount of heterogeneity was always less than in the heart, but seemed more variable between individuals. From some individuals, no product could be amplified from skeletal muscle DNA at all, even though a single copy nuclear amplicon was efficiently synthesized in all cases, as an internal control. From kidney DNA (as well as brain; data not shown) a heterogeneous set of products was amplified from all individuals, although its exact profile was somewhat variable. Sperm DNA gave single species representing sublimons of 3754, 3762 and 3784 bp, but the relative levels of these three species varied considerably between individuals. The same primer pair consistently gave no product at all with template DNAs from lung, pancreas or blood of multiple individuals, even though control amplicons were efficiently synthesized from these templates. Very minute amounts of product were evident in liver or skin of some individuals.

Sublimon abundance varies between <0.1 and >100 copies per cell

In order to estimate the abundance of sublimons of the prevalent (3.75 kb) class, we carried out multiplex PCR using primers across the deletion junction, combined with primers for a single copy nuclear gene giving a similarly sized PCR product (172 bp) as an internal standard. The products, labelled by two different fluorescent labels, were analysed by capillary electrophoresis at increasing cycle times to ensure that the reactions had not yet reached saturation. Data from any reaction points where saturation was inferred were discarded from analysis. Ratios were computed of the amount of nuclear and sublimon product at each sampling point. To guard against artefacts arising from impurities in different template preparations, we diluted template DNAs 5- and 25-fold to check that the ratios obtained were not altered. The method is illustrated in Figure 6. The results shown in Table 2 are for a set of six control hearts and for various different tissues of other individuals, mainly collected post mortem.

Three general points may be extracted from these data. Firstly, sublimon abundance varied between tissues and between individuals over a range of at least three orders of magnitude, from 95 single copy equivalents, i.e. 190 copies per cell down to <0.1 copies per cell (but still detectable). Secondly, in any individual, a clear and similar pattern of relative abundances in different tissues was evident, regardless of the absolute levels present: heart > muscle > brain ≈ kidney > skin ≈ liver, with no detectable sublimons of this class detectable in the pancreas, in the lungs or in the blood. Thirdly, sublimon abundance showed no simple relationship with age. Thus, the 22- and 77-year-old males surveyed both had low levels of sublimons in all tissues, whereas a second male of

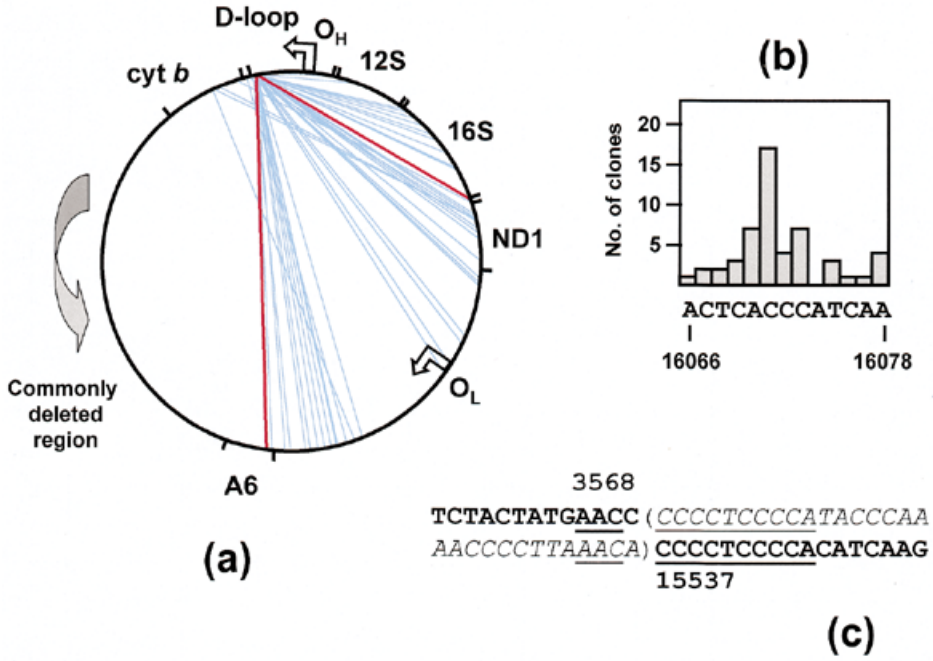

Figure 3. Sublimons detected in heart DNA by long PCR, cloning and sequencing. (**a**) Schematic diagram of human mtDNA showing the deletions detected (for details see Table 1). Faint blue lines join the break-points of the various deletions, all of which eliminated the region following on anticlockwise from the D-loop and cytochrome *b* (cyt *b*) gene as shown. Bold red lines indicate the two rearrangements that were each found in many different clones (Table 1), i.e. the prevalent 3.75 kb rearrangement between the CBR and nucleotide positions 3255–3272, in the gene for tRNA-leu^{UUR} lying between those encoding 16S rRNA (16S) and NADH dehydrogenase 1 (ND1) and the 7.4 kb deletion between the CBR and nucleotide positions 8636–8648 in the gene for ATPase 6 (A6). The positions and orientations of the replication origins for the two strands, O_H and O_L, based on the original strand-asymmetric mtDNA replication model, are indicated by arrows. (**b**) Bar chart showing the prevalences of the different break-point nucleotides within the CBR, inferred from sequencing. The numbers represent the first nucleotide pair in each sublimon retained on each side of the break-point within the non-deleted region. Where short repeats created ambiguity regarding the exact break-point, the first nucleotide (lowest numbering) was taken in every case. (**c**) A typical example of a short direct repeat across deletion break-points (from clone 38). The break-points occur within an 11 bp direct repeat, which extends by a further 4 bp beyond the breakpoint, if a 1 bp mismatch is ignored.

80 years of age had very high levels, especially in heart and skeletal muscle. One other skeletal muscle sample from an aged person (82-year-old female, s10), had very high levels of the prevalent sublimon class (190 copies per cell). In addition, we were unable to detect the prevalent 3.75 kb sublimon class in muscle, heart, brain or liver DNA samples from paediatric subjects. The number of sublimons of the prevalent (3.75 kb) class can clearly reach levels representing at least several per cent of total mtDNA. Note, however, that this represents a set of at least 20 and potentially hundreds of different molecular species, because of microheterogeneity at both of the break-points. Each individual sublimon is therefore present at most as a few copies per cell and usually much less.

Sublimons represent partially duplicated and deleted mtDNA

The above analyses do not distinguish between the various molecular forms carrying the same primary rearrangement (Fig. 1). To investigate this, we designed PCR primers capable of amplifying across the potentially duplicated region (Fig. 7). Primers X and Y, lying just within the deleted region, generated long PCR products of 3.8, 7.6 and ~11.5 kb using heart DNA templates in which the prevalent sublimons were easily detectable by PCR.

The larger products are exactly as predicted for partial duplications and triplications and cannot be generated from deleted molecules. The junctional primer Z, extending by just 6 nucleotides across the break-point of sublimons with the exact sequence of clones 19 and 22, gave the predicted, duplication-specific product of 3.8 kb, when used in combination with primer X on sublimon-containing template DNA. Together with primer W, lying just within the deleted region, primer Z gave the predicted sublimon-specific 3.6 kb product. The identity of these products was confirmed by direct sequencing. Neither can be generated from wild-type mtDNA. A myocardial DNA template in which significant levels of sublimons could not be detected did not give these products.

These findings indicate that a proportion of the prevalent sublimons exist as partial duplications and probably even as triplications, although the presence, in addition, of deletion monomers and multimers cannot be excluded. Primer pair XZ (but not WZ) also gave a faint band in the region of 7.5 kb, that may derive from deletion dimers. In order to demonstrate more clearly the presence of deletion monomers and multimers, we used a Southern blotting approach. This was technically demanding, since the background smear from the many thousands of wild-type mtDNA molecules per cell completely obliterated any signal from the low percentage of molecules with the anticipated structures, even when using probes

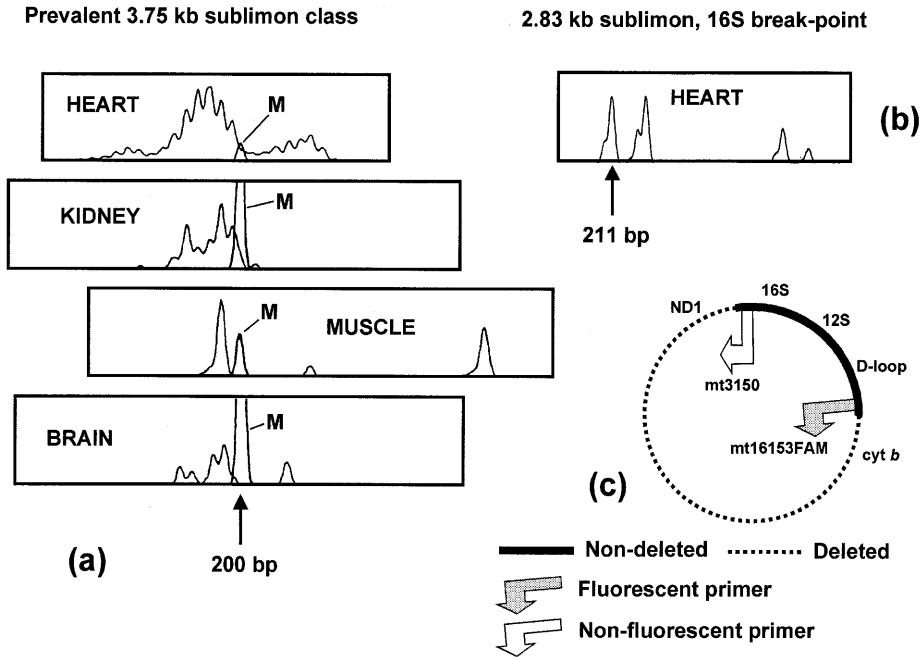

Figure 4. Fluorescent PCR analysis of the representation of specific sublimon classes in different tissues of a single individual (s5, 61-year-old male) post mortem. The labelled primer was mt16153FAM, adjacent to the CBR and the respective unlabelled primers were (**a**) mt3150 and (**b**) mt2204, used as illustrated in (**c**). These detect, respectively, (a) sublimons of the prevalent 3.75 kb class, as PCR products in the 185–225 bp size range and (b) sublimons of 2.83 kb, equivalent to those represented as clones 3, 13 and 17, with a break-point in the 16S rRNA gene (Table 1), as PCR products in the 210–230 bp size range. M, 200 bp size marker. Panels shown are outputs from GeneScan analysis carried out using the Applied Biosystems 310 capillary electrophoresis instrument, which resolves to the 1 nucleotide level.

specific for the non-deleted region. We therefore probed such blots using a junctional oligonucleotide. However, to obtain significant signal, we found it necessary to reduce the stringency of hybridization to the point where hybridization to undeleted mtDNA was still unavoidable. Nevertheless, under these conditions we did reproducibly detect low molecular weight bands uniquely in those myocardial DNA samples previously shown by PCR to have a high sublimon content (Fig. 8). These were not easily detected in undigested DNA, where the majority of mtDNA apparently migrated as higher order, probably catenated structures. After digestion with a restriction enzyme that cuts mtDNA only once, in the deleted region, bands were detected that correspond in mobility with supercoiled circles of the sizes expected for deletion monomers (3.75 kb), deletion dimers (7.5 kb) and trimers (11.5 kb). None of these species was detected by a probe for the *ND4/ND5* region of mtDNA. A *Bam*HI-linearized duplication in the 20 kb range would migrate close to the 16 kb linear band, hence could not easily be detected by this test.

Under similar hybridization conditions, digests predicted to cut once in the non-deleted region, for example *Dra*I (Fig. 8d) or *Afl*II (data not shown), gave only the 3.75 kb linear monomer band of the sublimon, as well as the D-loop containing fragment from wild-type mtDNA.

The bands proposed to represent the circular deleted mtDNAs were undetectable in all other tissues, including even muscle, where the predominant sublimon of 3762 bp is 2 bp shorter than that represented by the oligonucleotide used and has a junctional sequence that is probably too poorly matched to cross-hybridize efficiently. DNAs from ρ^0 cells that were probed with the oligonucleotide were completely blank.

In summary, PCR and Southern blot analysis revealed that myocardial sublimons with a precisely defined junctional sequence can exist in a variety of molecular species, with properties consistent with the various interconvertible forms illustrated in Figure 1.

DISCUSSION

In this study we set out to analyse the structure and representation of rearranged mtDNA molecules in healthy human tissues and to determine the relationship between such molecules and those found in association with pathological states. To describe them we have adopted the term sublimon, previously used to describe rearranged mtDNA molecules present at low abundance in mitochondria of healthy maize plants (24), with structures akin to those represented much more abundantly in strains exhibiting the mitochondrial cytopathy of cytoplasmic male sterility. Our analysis reveals features of human mtDNA sublimons that are very similar to those characteristic of pathological rearrangements, plus others that seem to be more specific.

Tissue specificity of sublimons

The spectrum of sublimons that appear to be present is strikingly tissue specific, at both the gross- and fine-structure levels. In general, post-mitotic tissues such as muscle or brain contain higher levels of sublimons than perennial tissues such

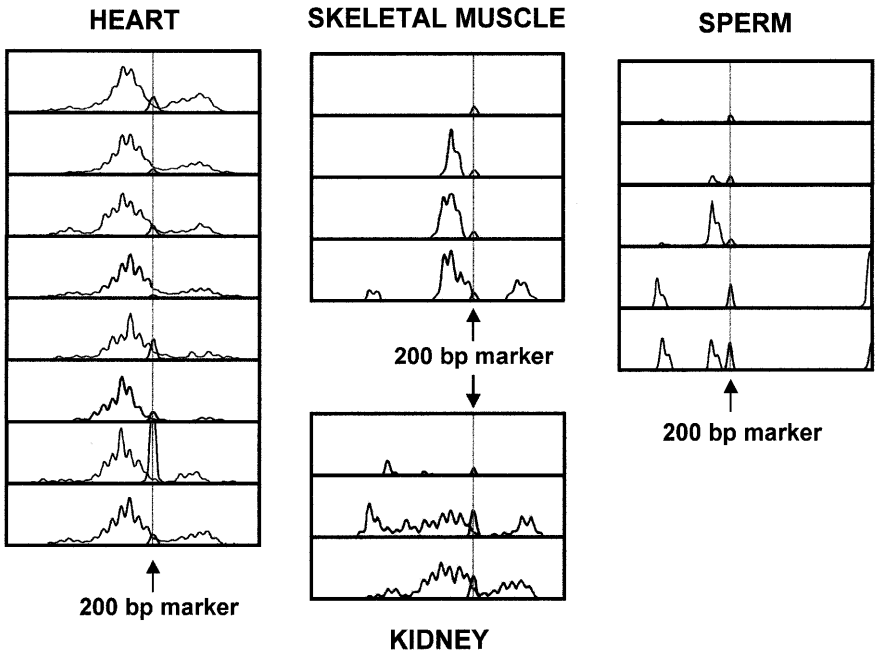

Figure 5. Fluorescent PCR analysis of the prevalent 3.75 kb sublimon class in different tissues of multiple control individuals. Primers were as in Figure 4a. Panels shown are outputs from GeneScan analysis resolved at the 1 nucleotide level. Heart, skeletal muscle and kidney DNAs were from various healthy individuals post mortem. Sperm DNA was obtained from samples collected for routine analysis in a fertility clinic, from five individuals known to be fertile.

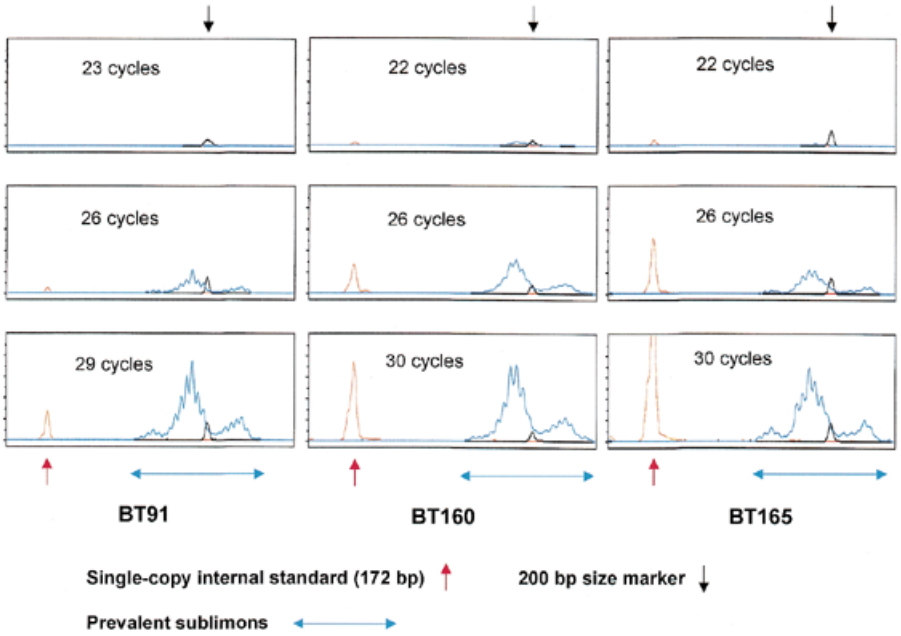

Figure 6. Semi-quantitative, multiplex fluorescent PCR analysis of the prevalent 3.75 kb sublimon class in heart DNA samples extracted from three different control individuals without coronary heart disease or any other disease affecting myocardium (BT91, BT160 and BT165) (Table 2). The blue channel shows product from sublimon primers as in Figure 4a, and the red channel the single copy nuclear DNA control (a cytochrome P450 gene). Where appropriate, DNA templates were diluted after test reactions, so as to give approximately equal sublimon signals after 20–30 cycles, in order to avoid saturation artefacts in cases where sublimons were highly abundant. The outputs from GeneScan analyses, as shown here, were used to compute the data shown in Table 2. Based on the incremental synthesis in successive cycles, the sublimon-specific and nuclear single copy primers gave comparable reaction efficiencies.

Table 2. Abundance of sublimons of the prevalent 3.75 kb class in tissues of different healthy individuals: copies per cell

Individual	Age (years), sex	Heart	Muscle	Brain	Kidney	Liver	Lung	Pancreas	Blood	Skin
s1	22, male	<0.1	8.2	2.5	0.6	0	0	0	0	0
s2	80, male	92	66	4.2	3.8	0.1	0	0	0	0.2
s3	75, female	3.7	2.7			0	0	0	0	0
s4	77, male	0.8		0.3	3.0	0	0	0		
s6	23 weeks[a]	0		0		0				
s7	28, female								0	
s8	65, male	12								
s9	73, male	1.9		1.1		<0.1				
s10	82, female		190							
BT91	49, male	31.8								
BT135	67, male	0.4								
BT152	46, male	2.2								
BT160	66, male	9.3								
BT162	33, male	<0.1								
BT165	56, male	3.9								

The values shown are mean ratios using the sum of peak areas for all of the sublimons detected by the primer pair used.
[a]Fetus, sex unknown.

as blood or skin and each tissue has a characteristic set of abundant sublimons. This could reflect different strengths and types of phenotypic selection operating in different tissues, as already implied by the observation of stereotypic shifts in heteroplasmy in different mouse tissues (26). Alternatively, it could indicate tissue-specific differences in the mode or machinery of mtDNA maintenance.

How far do sublimons resemble pathological rearrangements?

Within any given tissue, the spectrum of different sublimons is considerable. However, except for the most abundant species, all are present at low levels, typically of the order of 1–2 copies per cell or less. Even sublimons of the prevalent class, considered collectively, are rarely present at >50 copies per cell and usually much less. Putting together the data of Figure 2 and Table 2, we estimate that at most a few dozen different sublimons are ever represented at 1 copy per cell or more, with many others represented at significantly lower levels. The total number of sublimon molecules per cell is therefore typically of the order of 100 or less, in other words representing only a small percentage of the mtDNA population. In contrast, rearranged mtDNA molecules found in pathological states typically represent much larger fractions of total mtDNA, for example 30–70% for single clonal deletions or partial duplications and at least 10%, commonly much more, for multiple deletions, with some individual rearranged molecules representing up to 10% of total mtDNA in a given tissue.

Structurally, sublimons show a number of features in common with multiple deletions, and many deletions previously reported in diseased individuals (5,8,11,27) correspond precisely or almost precisely with those that we found in controls. The prevalent 3.75 kb sublimon class was also previously found as a partial duplication/triplication at high abundance in the muscle of an individual without overt disease (28). As was previously observed for multiple mtDNA deletions in multiple families with adPEO, MNGIE and IBM, the vast majority of sublimons show break-points in the region of nucleotide position 16 070, near the end of the D-loop (7,8,11,12,29). This CBR shows heterogeneity at the nucleotide level, but the other rearrangement break-points are more scattered, as is also the case in adPEO deletions. This leads to under-representation of the region adjacent to the end of the D-loop in the rearranged molecules (i.e. the region between the replication origins), as seen also in pathological deletions. A striking difference is that sublimons, at least in some tissues, for example heart, kidney or frontal cortex, mainly represent very large deletions lacking O_L, whereas pathological deletions generally retain O_L. However, because our analysis is based on PCR and plasmid cloning, techniques that both favour short products, it has an intrinsic bias in favour of very large deletions that may not reflect the true spectrum of sublimons present *in vivo*. This may account, for example, for the intriguing failure to find the common deletion, although it may not in any case be as common in heart as in skeletal muscle.

In sublimons as in pathological rearrangements (5,6), a single primary rearrangement can be associated with multiple molecular species that are theoretically interconvertible by homologous recombination (Fig. 1). The largest sublimon 'deletions' that remove O_L are clearly found also as partial duplications.

What mechanism generates sublimons?

The location of the common break-point region near the end of the D-loop suggests that sublimons may be generated by a recombinational mechanism: the free 3′ end of the paused

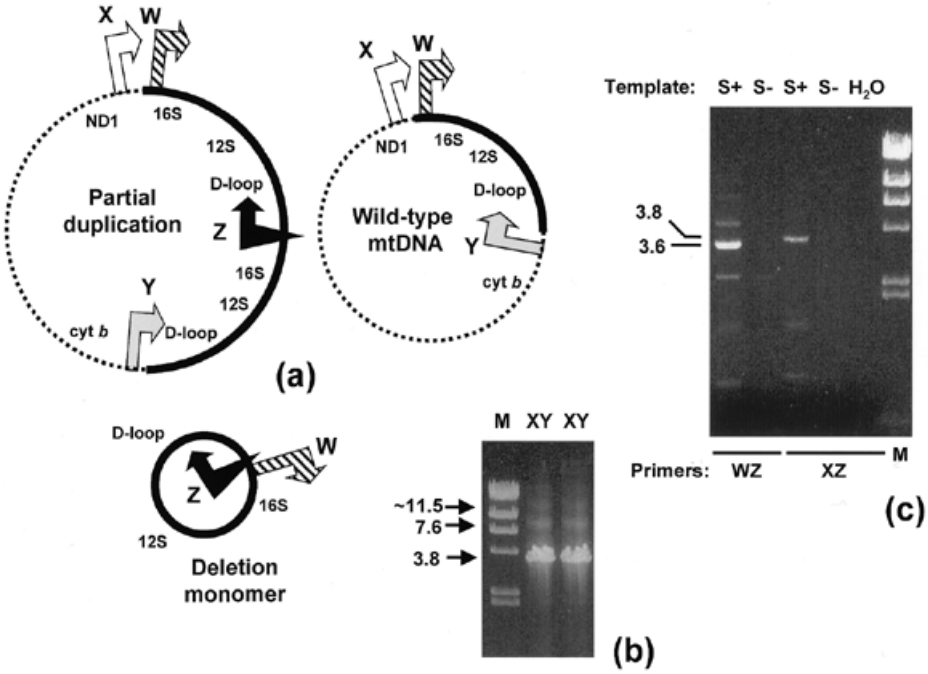

Figure 7. Detection of sublimons as partially duplicated mtDNAs, using long PCR. (**a**) Schematic diagrams of the various hypothesized rearranged mtDNAs carrying the same primary rearrangement (prominent 3.75 kb sublimon class). Nomenclature is as in Figures 1 and 3. Four different primers are shown: X (nucleotide positions 3321–3302 of human mtDNA) and Y (nucleotide positions 15 976–15 995) are in deleted DNA flanking the non-deleted region, W (nucleotide positions 3080–3060) is just inside the boundary of non-deleted DNA at the end of the 16S rRNA gene. Z is a primer across the rearrangement break-point of the sublimon represented as clones 19 and 22 (i.e. nucleotide positions 3247–3261 and 16 072–16 077 of human mtDNA), extending by just 6 nucleotides on the 3′ side of the break-point, thus ensuring that only rearranged mtDNAs can give the predicted amplification products. (**b**) Agarose gel analysis of long PCR products using primers X and Y on control adult heart DNA (M, markers). (**c**) Agarose gel analysis of long PCR products using primer pairs WZ and XZ on heart DNA templates that were previously found, based on sensitive fluorescent PCR assays as shown in Figures 4–6, either to contain (S+, healthy individual BT91) or not to contain (S–, healthy individual s1) significant amounts of the prevalent 3.75 kb sublimon class. Primer pair WZ gave the predicted 3.6 kb product from the sublimon-containing sample only, which also yielded the 3.8 kb duplication-specific product, using primer pair XZ.

H-strand could serve as an inappropriate primer for onward DNA synthesis, following strand invasion elsewhere in the genome, a mechanism already proposed in the case of pathological deletions (30). Even without readthrough of the origin, resolution of the resulting structure could generate either a deletion or a partial duplication. The second most commonly observed break-point hotspot region, at least in heart and skeletal muscle, is in the vicinity of the binding site for the transcription termination factor mTERF (31,32), within the gene for tRNA-leu^{UUR}. The termination region of the D-loop is also known to be bound by proteins (33–35). Illegitimate recombination events that generate sublimons might therefore be facilitated by protein–protein interactions, juxtaposing distant regions of the genome.

The tissue specificity of sublimons, even at the microscopic scale, could therefore arise from systematic tissue differences in the properties or representation of mtDNA-binding proteins, some of which may also induce replication pausing (36), to generate specific 3′ ends. *In vivo* footprinting studies have revealed a similar microscale variation in the effects of protein binding on the state of mtDNA, for example in response to thyroid hormone (37), or the different stages of early development in sea urchins (38). Because different sublimons are present in different tissues, in some cases at <1 copy per cell, they cannot be inherited and must instead be generated *de novo* somatically, perhaps on a continuous basis.

Why do sublimons remain at low levels?

Previous studies have shown that high levels of deleted molecules are highly deleterious to oxidative function (39,40), whereas partial duplications have only very modest effects on phenotype (40,41). However, despite the replicative advantage of partially duplicated molecules carrying supernumerary copies of the replication origin (41,42), their accumulation should be balanced by homologous recombination, resolving them to wild-type mtDNA and deleted molecules that are then counterselected phenotypically (41). Deleted species lacking O_L may be lost even without phenotypic selection, as a result of inefficient replication. Although the idea that the light-strand origin O_L is strictly necessary for mtDNA replication may not apply universally (43), its presence is nonetheless likely to confer a replicative advantage. Pathological duplications can indeed resolve in cell culture to wild-type and deleted mtDNAs, but the latter typically remain at a lower level (42) and in some cases are undetectable.

We therefore suggest that sublimons and wild-type mtDNA probably co-exist in an equilibrium condition in which sublimons are continuously lost and regenerated, but never accumulate to significant levels except under highly abnormal conditions. The exact position of this equilibrium may vary, however, even between healthy individuals, since sublimon abundance appears to vary over a considerable range even in individuals with no known history of systemic disease.

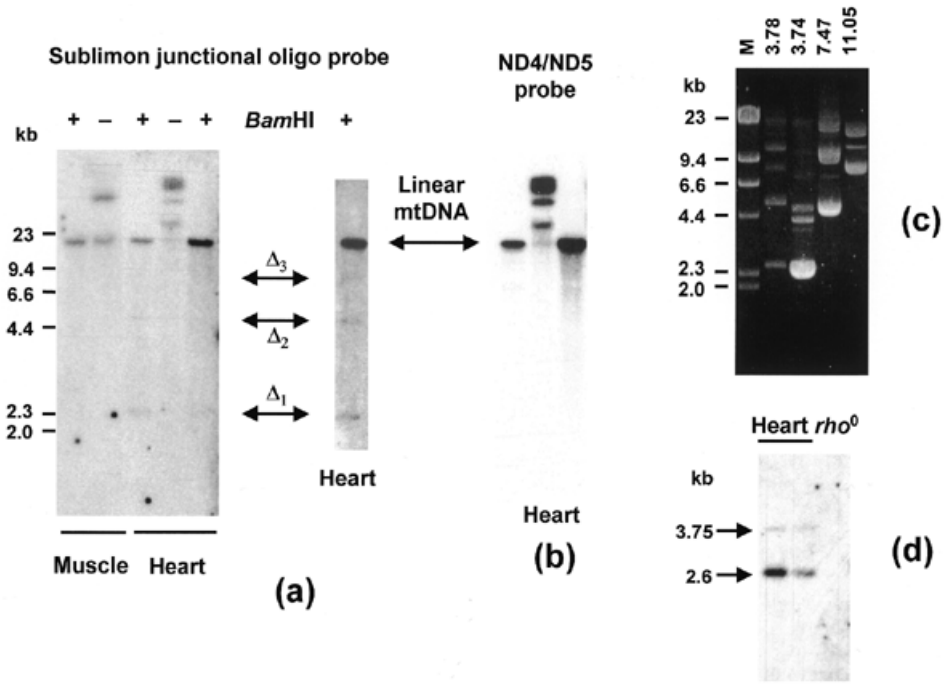

Figure 8. Detection of sublimons as monomeric and multimeric deleted mtDNAs, using Southern blotting. Blots of *Bam*HI-digested and undigested genomic DNA from heart (two different individuals) and skeletal muscle that had already found to contain prominent sublimons of the prevalent 3.75 kb class, probed (**a**) with the sublimon junctional oligonucleotide (Junc-1) and (**b**) after stripping, with a PCR product for the *ND4/ND5* region of mtDNA as described previously (14). Arrows indicate the positions of linear mtDNA (16.6 kb), plus bands proposed to correspond with uncut circles of monomeric, dimeric and trimeric deleted mtDNAs, most clearly seen in digested samples from heart, in the longer exposure shown separately. (**c**) Agarose gel electrophoresis of supercoiled circular plasmids of 3.74, 3.78, 7.47 and 11.05 kb, plus their respective multimers, showing the correspondence in migration between the sublimon-specific bands detected in (a) and circles of the expected size for deletion monomers (3.75 kb), dimers (7.5 kb) and trimers (11.25 kb). M, molecular weight marker (λ *Hin*dIII digest). (**d**) *Dra*I digests of two preparations of heart DNA and DNA from ρ^0 cells, probed with the junctional oligonucleotide Junc-1.

Could sublimon amplification be a pathological mechanism?

Given that at least some sublimons resemble pathological rearrangements, for example the multiple deletions seen in such conditions as adPEO, we need to re-evaluate thinking about the mechanism underlying the emergence of rearranged mtDNAs in pathological states. Instead of invoking a rare, spontaneous aberration in DNA replication or repair, it may be more appropriate to regard pathological rearrangements as illegitimately amplified sublimons. This suggests three new classes of mechanism by which rearranged mtDNAs could accumulate to physiologically damaging levels: (i) via an enhancement of the process(es) by which sublimons are continuously generated; (ii) via inhibition of the process(es) by which they are usually lost; and (iii) by a (possibly transient) alteration in their selective value, whether at the level of phenotype or replicative advantage.

If replication pausing, strand invasion and protein binding are involved in the generation of sublimons, any change or abnormality in the rate or processivity of DNA replication, the level and activity of mtDNA-binding proteins or the efficiency of lagging strand initiation could shift the equilibrium in favour of sublimon accumulation. This is strongly suggested by the observation that mutations in either of two genes whose products are involved in nucleotide metabolism, namely thymidine phosphorylase (44) and one isoform of the mitochondrial adenine nucleotide translocator (45), have recently been shown to cause multiple deletion disorders (MNGIE and one type of adPEO, respectively). Subtle differences in the spectrum of rearranged mtDNAs in different disorders (10) may give clues as to the precise mechanisms by which sublimons become amplified in pathological states. Since recovery from mtDNA depletion appears to involve a different mode of mtDNA replication from that operating in proliferating cells maintaining a stable mtDNA copy numbr (43), transient or periodic copy number aberrations may be sufficient to promote sublimon accumulation. It has also been proposed that deleted mtDNAs could affect the lysosomal turnover of mitochondria, by virtue of effects on the rate of oxygen radical generation (46); if this is the case, pathological sublimon amplification might be a consequence of abnormalities in this process, rather than anything specifically affecting mtDNA metabolism.

Sublimons and ageing

Our attention was first drawn to sublimons because of the use, by others, of long PCR to detect deleted mtDNAs that were purported to accumulate in the course of somatic ageing and in association with acquired myocardial diseases. We demonstrated earlier that the sensitivity of the technique is so great that it can detect rearranged molecules present at only one or a

few copies per cell, even against a background of thousands of molecules per cell of 'wild-type' mtDNA (17), an assertion supported by the analysis presented here. This raises considerable doubts as to the significance of reports linking deleted mtDNAs to ageing.

However, sublimon levels clearly vary between individuals and it cannot be excluded that they might accumulate under some conditions, even in an essentially healthy person, and could contribute to age-related degeneration. Our findings indicate that there is no simple relationship between age and sublimon abundance. A careful and rigorously controlled study is now required to evaluate whether sublimon amplification does occur at the expense of normal mtDNA in at least some individuals or tissues and whether this has any phenotypic consequences.

MATERIALS AND METHODS

Patients and tissue samples

Frozen samples of heart muscle were obtained from medico-legal autopsies performed in 1991–1992. These comprise five cases of alcoholic cardiomyopathy (see legend to Fig. 2), originally collected for other studies, plus controls excluded from having any heart disease or any known history of alcohol abuse, as previously (14). Paediatric biopsy samples (11 skeletal muscle, 4 heart, 2 liver, 1 brain from children from 14 weeks gestation to 14 years) were collected in Tampere University Hospital (Finland) and the John Radcliffe Hospital (Oxford, UK) from individuals without diagnosed mitochondrial or other relevant systemic disease. Muscle biopsies from six adult controls (ages 18–82) were taken at the Neurology clinic in University of Oulu (Finland) or in Oxford. Post-mortem tissue samples (time from death to autopsy 2–3 days) were taken from each of six other individuals (s1–s6) during the routine autopsy protocol (for the selected tissues see Table 2) and the DNA was extracted immediately after sampling. Control sperm samples from anonymous males known to have fathered children were kindly provided by Dr L. Wichmann (Department of Anatomy, University of Tampere, Finland).

Cell lines

143B osteosarcoma-derived and A549 lung carcinoma-derived ρ^0 cells (lines 206 and B2, respectively) (47,48) were cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum, supplemented with pyruvate and uridine (47).

DNA extraction

DNA was prepared by standard methods as previously (14), essentially by proteinase K digestion and phenol–chloroform extraction, from tissue samples and cell lines.

Oligonucleotide primers

Custom-designed primers for PCR and DNA sequencing were purchased from DNA Technology (Aarhus, Denmark) or Genset (Paris, France). The adjacent primer pair OK1H/OK2L, located in the mtDNA control region and primer FR31H from within the *COXI* gene, were as described previously (14). Additional PCR primers used in the study, all given as 5′→3′ and, where appropriate, followed by the corresponding co-ordinates in the human mtDNA sequence (49), are as follows: mt3150 (5′-TACTTCACAAAGCGCCTTC-3′, 3150–3168), mt2204 (5′-TTCAAGCTCAACACCCACTA-3′, 2204–2223), mt16153FAM (5′-CAGGTGGTCAAGTATTTATGG-3′, 16153–16133, incorporating a 5′ FAM fluorescent label), W (5′-CCGGTCTGAACTCAGATCAC-3′, 3080–3060), X (5′-GTTGGCCATGGGTATGTTGT-3′, 3321–3302), Y (5′-TCCACCATTAGCACCCAAAG-3′, 15 976–15 995) and Z (5′-GGTAATCGCATAAAACCATCA-3′, 3247–3261 and 16 072–16 077). PCR primers for cytochrome P450 2E1 were: DraIR (5′-TCCCAAAGTGCCAGGATT-3′) and DraIFROX (5′-ATCATGGCTCATTGTAGCTTC-3′, incorporating a 5′ ROX fluorescent label). Oligonucleotide Junc-1 (5′-GGTAATCGCATAAAACCATCAACAACCGCT-3′, nucleotide positions 3247–3261 and 16 072–16 086 of human mtDNA) was used as a hybridization probe. In addition, a series of sequencing primers corresponding with appropriate portions of the human mtDNA sequence were designed for analysis of cloned sublimons.

Long PCR

Reactions were carried out using primer pair OK1H/OK2L and analysed by agarose gel electrophoresis exactly as described previously (14). The amounts of template DNA were as indicated in the legend to Figure 2. For cloning, scaled-up long PCR reactions used 26 ng of a control heart DNA template (individual BT91, 49-year-old male) that had given diverse and abundant sublimon products. For cloning the largest sublimons (i.e. those with the shortest deletions) PCR was carried out using 'sub-genomic' primer pair FR31H/OK2L (14). Identical products were obtained using nested PCR, on gel-purified products of the first PCR reaction, migrating in the 7–15 kb size range.

Molecular cloning and sequencing

Long PCR products were cloned into pCR-XL-TOPO vector (Invitrogen BV, Groningen, The Netherlands) by the manufacturer's recommended protocol. Sixty-five clones (42 obtained by PCR using 'genome length' primers, plus 23 derived using 'sub-genomic' primers) were selected, sized by miniprep analysis and sequenced using BigDye terminator chemistry (Applied Biosystems, Foster City, CA), with a combination of vector-specific and mtDNA-specific primers. Sequencing products were analysed by capillary electrophoresis on an Applied Biosystems 310 Genetic Analyzer, using the manufacturer's software.

Fluorescent PCR

Fluorescent PCR to characterize specific sublimon subclasses used fluorescent primer mt16153FAM, plus either of primers mt3150 or mt2204. For semi-quantitative analysis, multiplex reactions included also primers DraIR and DraIFROX as an internal single copy gene standard. After a manual hot start for 3 min at 95°C, a PCR cycle consisting of 30 s at 95°C, 20 s at 59°C and 20 s at 72°C was repeated 22–30 times and the fluorescent products were analysed by capillary electrophoresis using GeneScan software on an Applied Biosystems 310 Genetic Analyzer. An additional 7 min final extension step to maximize the 3′ A-overhang addition efficiency did not affect the relative or absolute amounts of the fluorescent products in

any sample tested. Reaction products resolved at the nucleotide level were quantified as peak areas in the electrophoretogram and ratios computed of the amount of sublimon product versus the single copy standard, at increasing cycle number. Data from points where saturation had not yet occurred were pooled, to extrapolate a mean copy number per cell, i.e. twice the number of single copy equivalents, for all sublimons of the prevalent 3.75 kb class detected by the primers, considered collectively.

Southern blotting

Aliquots (5 or 10 µg) of genomic DNA, with or without *Bam*HI digestion, were fractionated by 0.7% agarose gel electrophoresis in the absence of ethidium bromide. Southern blotting, hybridization and washing were as described previously (14). The sublimon junctional oligonucleotide Junc-1 was end-labelled with [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, UK) using T4 polynucleotide kinase (MBI Fermentas, Vilnius, Lithuania) in the supplied reaction buffer A, according to the manufacturer's instructions, before addition to the hybridization reaction. Hybridization was carried out in a standard buffer (14) at 50°C for 16 h after a pre-hybridization step of 1 h at 50 °C without the probe. The blot was washed for 10 min in 5× SSC, 0.1% SDS, 0.025 M sodium phosphate pH 6.8 and for 5 min in 1× SSC, 0.1% SDS, 0.025 M sodium phosphate pH 6.8, both at 50°C. The radioactive signal was detected by autoradiography. Stripping and reprobing with a probe for the *ND4*/*ND5* region of human mtDNA were as described previously (14).

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