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Analysis of Distribution in the Human, Pig, and Rat Genomes Points toward a General Subtelomeric Origin of Minisatellite Structures

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We have developed approaches for the cloning of minisatellites from total genomic libraries and applied these approaches to the human, rat, and pig genomes. The chromosomal distribution of minisatellites in the three genomes is strikingly different, with clustering at chromosome ends in human, a seemingly almost even distribution in rat, and an intermediate situation in pig. A closer analysis, however, reveals that interstitial sites in pig and rat often correspond to terminal cytogenetic bands in human. This observation suggests that minisatellites are created toward chromosome ends and their internalization represents secondary events resulting from rearrangements involving chromosome ends.

INTRODUCTION

Tandem repeats are classified, according to the overall size of the repeat sequence, among satellites (largest, megabases long), minisatellites (in the kilobase size range), and microsatellites (10 to 40 nucleotides long). Satellite DNA is preferentially associated with centromeric heterochromatin, and microsatellites are distributed throughout the genome. Similarly, in the mouse (Jeffreys et al., 1987; J ulier et al., 1990; Mariat et al., 1993), bovine (Georges et al., 1991), and rat genomes (Pravenec et al., 1996) at least, minisatellites are spread over the genome. In contrast, the minisatellites characterized so far in human show a marked clustering in the terminal cytogenetic bands of each chromosome (Royle et al., 1988; Wells et al., 1989; Vergnaud et al., 1991).

This apparent discrepancy may be an artifact due to the cloning procedures that have been used: clustering in human is suggested by a variety of independent approaches performed in different laboratories, whereas the work in the mouse, rat, and bovine relies in each case essentially on a single approach. Furthermore, a very limited number of the supposed minisatellites detected (Jeffreys et al., 1987; J ulier et al., 1990; Mariat et al., 1993) or cloned (Georges et al., 1991) from animal genomes have been characterized at the sequence level to confirm their minisatellite nature. Alternatively, this discrepancy could reflect differences in the evolutionary forces that shape mammalian genomes.

To investigate this issue, we cloned minisatellites in human, pig, and rat using identical techniques and compared their chromosomal localization in the three genomes. Clones containing minisatellites were identified in genomic libraries using both synthetic tandem repeats as screening probes (Vergnaud et al., 1991) and a new procedure that is based only on the structural characteristics of the minisatellites and does not require a hybridization step. The cosmid were assigned to a cytogenetic band by in situ hybridization assisted when appropriate by linkage analyses. Striking differences were observed in the distribution patterns of these sequences, with subtelomeric clustering confirmed in human, a majority of internal loci in the rat,
and an intermediate situation in pig. The comparative mapping of the minisatellite-containing regions in
the three genomes is consistent with the hypothesis that
minisatellites have a subtelomeric origin. These re-
results underline the interest of minisatellites for
comparative genome analysis.

MATERIALS AND METHODS

Minisatellite Cloning

Cosmid clones from commercially available libraries (Clontech Refs. HL 1145y, RL1032m, and PL1008m for human, rat, and pig, respectively, on pWE15 vector) were plated at a low density and picked in 96- or 384-well microtiter plates. Replicas were made on
yylon filters with a comb.

Procedure without hybridization screening. The clones were
grown individually in 2-ml LB medium cultures with shaking at
37°C. After 20 h growth, 24 cultures were pooled, and the cosmids
were digested with two frequently cutting restriction endonucle-
ases. The alkaline SDS lysis method from 50 ml MLS (Sambrook
et al., 1983) was used to digest the cosmids. The alkaline SDS lysis method as described in
Sambrook et al. (1989). The cosmid DNA pools were extracted by

Procedure with hybridization screening. The clones were
grown individually in 2-ml LB medium cultures with shaking at
37°C. After 20 h growth, 24 cultures were pooled, and the cosmid
DNA was digested with two frequently cutting restriction endonucle-
ases. The alkaline SDS lysis method from 50 ml MLS (Sambrook
et al., 1989) medium culture and suspended in 300 µl of TE. In both procedures, three aliquots of 20 (for pools of clones) or 1 µg (for individual clones) of
cosmid DNA were digested respectively with the restriction endonucle-
ases AluI and HaeIII, AluI and Hinfl, and Hinfl and HaeIII in a total
volume of 100 µl following the suppliers instructions (New England
Biolabs or Appligene). After an overnight digestion, the samples were
ethanol precipitated, suspended in 5 µl of TE, and loaded for electrophoresis on a 1% horizontal agarose gel. The gel was then stained with
ethidium bromide. Fragments above 1.5 kb (from pools) or above 1kb
(from individual clones) were sliced out of the gel and recovered by
centrifugation as described by Heery et al. (1990). One to five nano-
grams of insert DNA was then directly [32P]-labeled by random priming
(Feinberg and Vogelstein, 1983). [32P]dCTP from New England Nu-
dea, random priming kit from Boehringer and hybridized at 65°C as
described in Vergnaud et al. (1991) to a Southern blot carrying the DNA
two from unrelated reference individuals digested by HaeIII, Hinfl, and
PvuII. This provided an identification pattern for each minisatellite
probe, and the profile was encoded by sizing each allele in the three
digests. In the case of a pool of clones, the cosmids from which a fragment
originated was identified by hybridizing back to the replica of the 384-
well plate.

Chromosomal Mapping by Linkage Analysis

Polymorphic loci were mapped by linkage analysis using the CEPH
panel of human families, the PIGMAp panel of pig reference families,
and a F2 (BN × GK) rat population (Gauguier et al., 1996). Geno-
types were managed using GENBASE, developed by Jean-Marc Se-
baun. Linkage file output from the human or pig data was converted to
CRIMAP file format using the LINK2CR1 utility software written by
John Attwood. CRIMAP version 2.4 was used for the analyses.

Fluorescence in Situ Hybridization

Rat chromosomal were prepared from normal rat fibroblast cul-
tures. The probes were labeled by nick translation with digoxigenin-
-11-dUTP (Boehringer Mannheim) and hybridized at a final concen-
tration varying from 2 to 10 ng/ml in presence of an excess of
sonicated rat DNA (between 100 and 300 ng/ml) used as competitor.
A standard hybridization was performed, as described in Apiou et al.
(1996). Immunochromic detection of hybridization was performed
using mouse anti-digoxigenin antibodies and sheep FITC-conju-
gated anti-mouse antibodies (Boehringer Mannheim). Rat meta-
aphases were stained with DAPI, and chromosomes were identified
with computer-generated reverse DAPI banding. Metaphases were
observed under a fluorescence microscope (DMRB, Leica, Germany).
Images were captured using a cooled Sensys CCD camera and Quips-
Smat capture software (Vysis).

Pig metaphase chromosome spreads were obtained from periph-
eral blood lymphocyte cultures by standard procedures. Chromo-
somes were G-banded after hybridization with the G-T-G tech-
nique, and pictures were taken using a video printer (Mitsubishi).
They were classified according to their G-band pattern as defined by
the Committee for the Standardized Karyotype of the Domestic Pig
(Gustavsson, 1988). Whole cosmids containing minisatellite sequences
were used as probes and were labeled by incorporation of biotinylated
16–dUTP (Boehringer Mannheim) by the random priming method
(Feinberg and Vogelstein, 1983). For suppression of background caused
by repetitive sequences, the cosmid probe (final concentration 2 µg/ml),
sonicated pig genomic competitor DNA (200 µg/ml), and salmon sperm
dna were ethanol precipitated together, dissolved in 25 ml of hybrid-
ization mixture, and prehybridized for 3 h at 37°C. In situ hybridization,
detection of the signals, and interpretation of the results were per-
formed as described previously (Yerle et al., 1992).

Minisatellite Subcloning and Sequencing

Minisatellite inserts were subcloned into PUC 18 or 19 and par-
tially sequenced from both ends using [33P]-labeled universal and reverse M13 primers and the cycle sequencing kit deltaTaq from
USB/Amersham.

Identification of Conserved Regions

Restriction fragments from pig cosmids were purified from agarose
gel as described before and used as probes to screen a human chro-
omosome 1 library obtained from the Max-Planck Institute for Molecu-
lar Genetics: c112 Reference Library, ICRF (Lehrach, 1990). This
library consists of two high-density gridded filters of 20,000 clones
on each membrane. Each clone is named by the number of the library,
c112, and a specific number (for example, c112 F0525). Hybridization
on the library filters was at 60°C as described by Vergnaud (1989).
Cross-hybridizing fragments from the human cosmids were identi-
fied by hybridization with the pig fragments on restriction digests of
human cosmids.

Conserved fragments from both species were subcloned in PUC 18
or 19 and sequenced as described before. Direct (universal) and
reverse M13 primers were used for sequencing of both ends, and
specific primers were ordered to continue the sequence reaction. The
accession numbers of the sequences generated in the course of this
project are AJ01151 to AJ01156.

RESULTS

Cloning of Minisatellites

Minisattelites were isolated from human, pig, and rat cosmid libraries by two procedures. The first pro-
cedure screens cosmid libraries with synthetic tandem
repeat probes (Vergnaud, 1989) and the second ("large
fragment procedure") does not need a hybridization
step (see Materials and Methods). Briefly, hybridiza-
tion-selected individual cosmids or pools of random
cosmids are digested with two frequently cutting re-
striction enzymes, and fragments above 1 (from hy-
bridization selected cosmids) or 1.5 kb (from pools of ran-
dom cosmids) are tested by Southern blot hybridization for
the presence of a minisatellite.
The number of minisatellites isolated with each
procedure is given in Table 1. Sixty-six, 34, and 43 mini-

satellites were isolated from human, pig, and rat libraries, respectively. The efficiency of the large fragment procedure varies from one species to another, with yields of 0.72% in human, 0.41% in rat, and only 0.2% in pig.

The efficiency of synthetic probes is different in the three species. Probes allowing the detection of minisatellites were 13C1, 14C21, 16C2, 16C4, 16C17, and 16C27 in human; 14C24, 15C3, 16C2, 16C7, 16C22, 16C23, 16C24, and 16C26 in pig; and 13C1, 14C3, 14C5, 14C31, 16C2, 16C4, 16C17, 16C20, 16C24, and 16C27 in rat. The most successful STR probes in the detection of minisatellites were 16C27 in human and 16C23 and 16C26 in pig, allowing the detection of 14, 6, and 6 minisatellites, respectively.

Identification of Minisatellite Structures and Sequences

The fragments detected were identified as minisatellites according to their hybridization pattern on Southern blots, i.e., a strong hybridization as expected for a tandem repeat probe. To confirm minisatellite structure, we subcloned and sequenced 20, 15, and 15 fragments from human, rat, and pig, respectively. A tandem repeat structure is observed in the three species for each fragment sequenced. In all but four cases, a repeat unit, with length ranging from 9 to 128 nucleotides, can be defined. Four fragments detecting multilocus hybridization patterns on Southern blots even under high stringency conditions are composed of successive di-, tri-, or tetranucleotide repeats interspersed with a large number of variant repeats. Consensus sequences of four minisatellites from each of the three species are presented in Table 2, together with the length of the repeat unit and internal variations among units.

The majority of variants is due to transition events (represented by letters Y and R). Transversions (S, K, or W) and sometimes three possible variants at the same position (represented by letters D, H, V, and B) are also observed. The number of variant sites in repeat units differs between the minisatellites from 2 variants/51 nucleotides in human minisatellite CEB84 and 30 variants/50 nucleotides in pig minisatellite ADGE S1. A length heterogeneity of the units resulting from insertion/deletion is sometimes observed [repeat (AGGG)n in CEB1].

Chromosomal Assignment of the Minisatellite Loci

The cosmids were assigned to a cytogenetic band by a combination of fluorescence in situ hybridization (FISH) and segregation analyses. This second approach was most appropriate for human loci because a physical assignment of highly polymorphic loci can usually be deduced from segregation data of CEPH families (Spurr et al., 1994) and the knowledge of the physical assignment of close-by markers (Bray-Ward et al., 1996) from the CEPH database. Thirty-nine, 33, and 34 cosmids were mapped by FISH in human, pig, and rat, respectively. The minisatellite localization is shown on Figs. 1A (hu-

### TABLE 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Method 1a (clones analyzed)</th>
<th>Method 2b (clones localized)</th>
<th>Total number of cosmids localized by FISHc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>36 (5000)</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>Pig</td>
<td>7 (3500)</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Rat</td>
<td>28 (7400)</td>
<td>34</td>
<td>34</td>
</tr>
</tbody>
</table>

a Method 1: screening with synthetic tandem repeat probes.
b Method 2: the large fragment procedure with no hybridization screening of clones (indicating total number of cosmids clones analyzed in pools).
c Number of clones localized by FISH.

### TABLE 2

<table>
<thead>
<tr>
<th>Namea</th>
<th>Unit sequenceb</th>
<th>Unit lengthc</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEB 1</td>
<td>GRGGAGGGAGGAGGGAGGG, TGG(C)CTGQGGAGGTCYGGYTGCA</td>
<td>37-43 bp</td>
</tr>
<tr>
<td>CEB 8</td>
<td>GGGRGGGTD</td>
<td>10 bp</td>
</tr>
<tr>
<td>CEB 62</td>
<td>ACCTACCCCRGGS(C)AGGAGSSAGGAGGCWGC</td>
<td>30-31 bp</td>
</tr>
<tr>
<td>CEB 84</td>
<td>GGTCACTATGGGGGTTGCTACAGGAACCAAGGGTTGCCACCCGCA</td>
<td>51 bp</td>
</tr>
<tr>
<td>ADGE S1</td>
<td>AYGWGDQYTBABYHCARTMTRKHTTYWKYRTGKWYTRAGYCYRRS(V)</td>
<td>49-50 bp</td>
</tr>
<tr>
<td>ADGE S2</td>
<td>GGATGCGGGRGGGACRGGG(R)CA</td>
<td>17-23 bp</td>
</tr>
<tr>
<td>ADGE S12</td>
<td>HAGITTCAGTTAGGTGVRG</td>
<td>19-20 bp</td>
</tr>
<tr>
<td>ADGE S40</td>
<td>T(A)GARRRJACGAGGCGWTGTCTCYTCAAGCGYGGGYTCY(T)C(Y)</td>
<td>35-40 bp</td>
</tr>
<tr>
<td>CEB R10</td>
<td>CCC(AAT)AT</td>
<td>5-8 bp</td>
</tr>
<tr>
<td>CEB R15</td>
<td>AYBRKGBH(C)T(J)CC</td>
<td>11-13 bp</td>
</tr>
<tr>
<td>CEB R21</td>
<td>AGRYRCCASRGYRYYRR</td>
<td>16 bp</td>
</tr>
<tr>
<td>CEB R31</td>
<td>DGCYCATCAGGGAGTYGCCT(G)AKTGAGA</td>
<td>28-29 bp</td>
</tr>
</tbody>
</table>

a Minisatellite name (CEB, human minisatellites; CEB R, rat minisatellites; ADGE S, pig minisatellites).
b Sequence of the elementary unit of the minisatellite loci. Variant positions are underlined. (R = A or G; Y = C or T; M = A or C; K = G or T; S = G or C; W = A or T; H = A, T, or C; B = G, T, or C; D = G, A, or T; N = A, G, C, or T; V = G, A, or C; IUB coding rules). The sequence presented is a consensus sequence deduced from the sequence of different motifs.
c Observed length of the different units.
FIG. 1. Chromosomal localization of minisatellites loci on human (A), pig (B), and rat (C) chromosomes (Giemsa banding). Minisatellites localized by both FISH and linkage analyses are represented by black circles. Minisatellites localized only by linkage analysis to physically assigned polymorphic markers are represented by open circles. The two loci represented at the tip of the short arm of the X chromosome are within the X–Y pseudoautosomal region and are also represented at the tip of Yp.
Gene assignments (Yerle et al., 1996). The proximal side of the fusion event is homologous to part of human chromosome 19, and the distal side is homologous to human chromosome 1p36.3. The lower proportion of subtelomeric minisatellites in pig (23 of 34; 66%) results from a few interstitial clustered or isolated loci (on chromosomes 5, 6, 7, 8, 10, 12, 13, and 17). In the rat genome, the situation is different, with a much lower rate of terminal band clustering (30%).

**Comparative Localization of the Minisatellites in the Three Genomes**

**Human–Pig Comparison**

Chromosome 6 alone accounts for 3 of the 11 interstitial sites detected in pig. Interestingly, the physical assignment of the minisatellite cluster at 6q2.1–q2.2 corresponds to the position of an ancestral chromosome fusion event with remnants of telomere arrays (Gu et al., 1996). The proximal side of the fusion event is homologous to part of human chromosome 19, and the distal side is homologous to human chromosome 1p36–p31 as deduced from heterologous chromosome painting experiments (Goureau et al., 1996) and a number of gene assignments (Yerle et al., 1997). We have identified conserved regions within cosmids containing the pig chr 6q minisatellites to characterize more precisely the homology between pig chr 6q minisatellite loci and human (Fig. 2).

The different fragments obtained after an EcoRI digestion of the four pig cosmids containing minisatellites ADGE S26, S37, S16, and S3 were used to screen a human chromosome 1 cosmids library at moderate stringency. Clones giving a hybridization signal with at least two fragments from the same pig cosmid and no signal with fragments from another cosmid were selected. No clone satisfying this criterion was obtained when fragments from the ADGE S26 cosmid were used. In the three other cases, cross-hybridizing fragments were identified and sequenced, and the human homologous cosmids were assigned to a chromosome 1 cytogenetic band by FISH.

Following is a characterization of the conserved sequences.

**Minisatellite Cloning**

We present here a differential organization of minisatellite loci from the human, pig, and rat genomes. The combination of two independent procedures was highly efficient for the cloning of these loci from genomic cosmid libraries. The method involving synthetic tandem-repeat hybridization probes has already proved its efficiency in detecting polymorphic loci in complex genomes (Mariat and Vergnaud, 1992) and was initially tested on a smaller scale for the cloning of such loci in human (Vergnaud et al., 1991). The species-specific efficiency of a given synthetic probe in minisatellite cloning in the human, rat, and pig genomes parallels our earlier observations on Southern...
blots of genomic DNA in a larger range of species (Mariat et al., 1992) and might indicate the existence of species-specific minisatellite families. The second method uses a repeat structure criterion and avoids the selection bias introduced by cross-hybridization. This method showed different isolation efficiencies for human, rat, and pig (0.7, 0.4, and 0.2%, respectively, of the cosmid clones analyzed; Table 1), suggesting that the frequency of minisatellites is highest in human or, alternatively, that on average human minisatellite alleles are longer.

Chromosomal Localization

Results reported here are in agreement with earlier ones conducted in human and suggesting minisatellite clustering in subtelomeric regions of human chromosomes (Royle et al., 1988; Vergnaud et al., 1991, 1993). Because the same methodology was here applied in a single laboratory to the three genomes, it is clear that the distribution of minisatellite localizations reported now is not an artifact introduced by cloning bias. Three different situations are observed, with 90% of subtelomeric origin of minisatellite structures...
meric clustering for human minisatellite loci, 66% for pig loci, and only 30% for rat loci. In addition, we demonstrate that minisatellites are not found equally at all chromosome ends. In this respect, human 1pter is most remarkable compared to 1qter. It may be relevant to note that 1p36 is a very strong T band (Dutrillaux, 1973) characterized by a high concentration of H3 isochores, CpG islands, and genes (Saccone et al., 1996; Zoubak et al., 1996). The much more homogeneous distribution of minisatellites observed in rat would then also be correlated with GC level, which is distributed more homogeneously in rodent chromosomes (Sabeur et al., 1993). The much more homogeneous distribution of minisatellites observed in rat would then also be correlated with GC level, which is distributed more homogeneously in rodent chromosomes (Sabeur et al., 1993). However, a number of discrepancies exist between the location of minisatellite clusters (for instance human 6qter is a minisatellite cluster; Fig. 1) and the location of T bands (6qter is not a T band; Dutrillaux, 1973). More generally, what is the reason for the relative paucity of minisatellites at some chromosome ends: does it reflect a more recent telomeric location or rather a specific behavior of some subtelomeric domains promoting the creation of minisatellites (either at a higher density or at a comparable density in a larger domain). Further studies and especially sequencing of a number of subtelomeric regions may help address these issues.

Comparative Analysis of Minisatellite-Containing Regions

Comparative gene mapping data available show a correspondence between pig interstitial minisatellite-containing regions and human chromosome extremi-
ties. The genes RNR2 and VWF (Von Willebrand Factor) localized respectively on pig chr 10p12–p13 and chr 5q21 (Miyake et al., 1988; Sjoberg et al., 1996) in the vicinity of interstitial pig minisatellites (Fig. 1) map on human terminal bands on chromosome 14p12 and chromosome 12p13.3, respectively (the VWF gene is also subtelomeric in cattle (chr 5q35), sheep (chr 3q35), and goat (chr 5q35); Janel et al., 1996).

Pig chromosome 6 results from a telomeric fusion between two chromosomes (Gu et al., 1996). Heterologous painting experiments (Rettenberger et al., 1995; Goureau et al., 1996) and comparative gene mapping reveal that the two extremities involved are homologous with human chromosome 1p and chromosome 19q (Fig. 2). Pig minisatellites ADGE S26 and ADGE S37 are localized in the band containing the telomere fusion between the two syntenic groups, and the human homologue of the ADGE S37 locus is indeed located in 1p36, showing that the pig interstitial chromosome 6 minisatellite duster is conserved syntenically with the human chromosome 1pter minisatellite cluster. Strikingly, the human homologue of the 6pter pig cosmids containing minisatellite ADGE S3 is located at 1p31–p32, from which we independently characterized a minisatellite locus (Fig. 1).

Although the situation in rat is expectedly much more complex, results from the localization of rat minisatellites provide further support for the hypothesis of a subtelomeric origin of minisatellite loci. As a general rule, comparative analysis between human and rat genomes is hampered by the paucity of mapped genes within the human chromosome 1p and chromosome 19q (genes Drd4 and Igf2) (Jeffreys et al., 1993; Paulsson et al., 1996), whereas microsatellites most likely mutate by replication slippage mechanisms, presumably initiated by double-strand breaks (Buard and Vergnaud, 1994; J effreys et al., 1994), whereas microsatellites have been duplicated. Interestingly, in the two independent examples reported in Wong et al. (1990) and Eichler et al. (1996), one of which contains a minisatellite, the ancestral site of the transposition event is telomeric (at respectively 6pter and Xq28) and both target sites are within band 16p11.

No general function has been attributed to minisatellites, but some of them do have a biological role. They can be coding sequences (Boguski et al., 1986; Paulsson et al., 1992) or regulatory elements (Krontiris et al., 1993; Bennett et al., 1995). The abnormal expansion of a minisatellite can create a fragile site (Virtanева et al., 1997; Yu et al., 1997). The analysis of neomutated alleles at other, nonpathological hypermutable mini- satellites has shown that they evolve predominantly by gene-conversion-like mechanisms, presumably initiated by double-strand breaks (Buard and Vergnaud, 1994; jeffreys et al., 1994), whereas microsatellites are most likely mutate by replication slippage mechanisms (Heale and Petes, 1995). Therefore, a good knowledge of domains in which minisatellites are likely to be found within the human genome may accelerate the identification of genetic disorders associated with this type of tandem repeat element.

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