Retroposons do jump: a B2 element recently integrated in an 18S rDNA gene

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ABSTRACT
Several cDNA clones were isolated from cDNA libraries constructed with mRNA longer than 28S RNA from the murine cell line PYS-2/12. The plasmids have inserts containing 1–1.2 kb of the ribosomal 5' external transcribed spacer followed by nearly 700 nt of sequence for 18S rRNA and ending with a B2 element (retroposon). The cloned sequence differed in a few positions from published ribosomal sequences. The 3' adjacent genomic sequence was obtained by polymerase chain reaction (PCR) and showed that the B2 element has a poly(A) tail of about 50 nt and is surrounded by perfect direct repeats of 15 nt. Analysis of genomic DNA from several murine cell lines revealed that PYS cells contain at least one copy of 18S RNA with the B2 element which is not present in the genome of other murine cell lines derived from the same teratocarcinoma. Similarly, rRNA transcripts containing the B2 element were only detected in PYS cells. According to the publication dates of the different cell lines, the B2 element must have been integrated into an rRNA transcription unit during the years 1970 through 1974 thus proving that retroposons (SINEs) can still be inserted into the genome in our times.

INTRODUCTION
Although much has been learned about transcription of rDNA genes by RNA polymerase I (reviewed in (1)) and although ribosomal RNA (rRNA) is the most abundant RNA in all cells, it has not been studied intensively in many aspects in mammalian cells.

According to current knowledge, all rRNAs are transcribed as a single precursor of 13.4 kb (47S RNA). Processing of the primary transcript was investigated in (2–4). It is generally believed that the first step in processing is cleavage at or near the 5' end of the 18S RNA and that the spacer segments are rapidly degraded. The DNA sequence of the 18S rRNA of mouse was established in (5, 6) and comprises 1869 nt. The sequences of the internal transcribed spacers (ITS) are given in (7, 8) as 989 and 1089 nt. The 5.8S and 28S rRNA were elucidated in (7–9) and consist of 157 and 4712 nt. The primary structure (4006 nt) of the 5' external spacer (ETS) was only recently completed (10). It is shortened by 650 nt as the first processing step (4). The 3' ETS is 565 nucleotides long (11). A schematic diagramm of the rRNA precursors and their lengths can be found in the Results section (Fig. 6A).

It has been shown that not all copies of human rRNA are identical as some base exchanges have been found between different clones (12, 13) but until now, no pseudogenes have been described for ribosomal genes in mammals.

We have isolated cDNA clones from poly(A)-enriched RNA from a murine cell line (PYS-2/12) which contain part of the 5' ETS as well as the 5' end of the 18S sequence and a B2 element (retroposon). We provide evidence that the clones are derived from transcripts of a ribosomal gene unit with the B2 element inserted into a copy of 18S rRNA which has to be regarded as a transcribed pseudogene. This newly inserted B2 element is specific for PYS cells.

B2 elements belong to the class of short interspersed repetitive elements (SINEs) that occur in about 10^5 copies in the genome of rodents (14–16). It is not known how SINEs are mobilized and, until very recently, no insertion of such elements in our time had been demonstrated (17, cf., 18, 19).

MATERIAL AND METHODS
Isolation of the cDNA clones
The construction of the cDNA libraries PG3 and cII has been described in detail (20). Library PG3 was screened with a synthetic oligonucleotide, 3NIU, complementary to the sequence 5' GCTGACTGCTTTGTGCTTGG 3' which was taken from the cDNA sequence of a clone coding for nidogen (21). The nylon filters containing the cDNA library were hybridized at 50°C in 5XSSC, 1% sarcosyl and washed at 38°C two times with 3 XSSC, 0.5% sarcosyl and two times with 3 xSSC. Plasmid DNA of about 20 positive clones was analyzed by restriction mapping to reveal longer inserts. Three plasmids, N1, N14 and N29, contained inserts of about 2 kb which were subsequently analyzed in detail.

The Stail fragment of plasmid N29 was labeled by the random priming method and used to screen a third of library cII. The cDNA sequence of a clone coding for nidogen (21). The nylon filters containing the cDNA library were hybridized at 50°C in 5×SSC, 1% sarcosyl and washed at 38°C two times with 3×SSC, 0.5% sarcosyl and two times with 3×SSC. Clones for nine strong signals were isolated and the plasmids exhibited similar restriction patterns. Three plasmids, N1, N14 and N29, contained inserts of about 2 kb which were subsequently analyzed in detail.

Sequence analysis
Suitable restriction fragments were subcloned either in M13tg130 or M13tg131 (Amersham) or the plasmid vector pUC830 (20). Parts of the original clones were sequenced directly or after
subcloning by the dideoxy chain termination reaction. All fragments were sequenced on both strands. Regions with strong secondary structure were sequenced in parallel with dGTP and dITP mixes (USB, sequenase kit).

Cell lines
Most murine cell lines have been described in detail in (22). GZ cells (mouse) were a gift of G. Lozano (23, 24). C1300 neuroblastoma cells (mouse) were obtained from the ATCC.

RNA isolation
Total RNA from different cell lines was prepared either according to (25) or (26) with some modifications (cf., 27). Recently the method in (28) was used for preparation of RNA from single culture dishes. Cytoplastic RNA was isolated according to (29).

Northern hybridizations
4 μg total RNA of the different cell lines were glyoxalated and separated on 0.7% agarose gels. The RNA was transferred to Hybond nylon membranes (Amersham). Further treatment of the blots is described in (27). The size of the RNAs was determined from glyoxalated DNA markers run on the same gels together with the RNA. The filters were hybridized and washed at 65°C with labeled M13 probes (30), containing different fragments of clone N29 (cf., 27).

The oligonucleotide, L18S, corresponding to the 3' sequence of the 18S part of the clones and the beginning of the B2 element (5' TCACCAGCCCAACTACGAGCTT 3') was labeled with 32P-gamma-ATP and hybridized to Northern blots in 5XSSC, 0.1% SDS, 0.1 xSSC at 65°C for at least 16 h. Before reusing the blots, probes were stripped off by incubation of the blots in 0.1% SDS, 0.1 xSSC at 65°C for at least 16 h.

Southern hybridizations
Genomic DNA from different murine cell lines was isolated either by lysing the harvested cells (32) or after preparation of nuclei (38). An arrow also marks the 3' border of the B2 element.

RESULTS
The cDNA clones presented here were isolated in an attempt to obtain clones for the basement membrane protein nidogen. The oligonucleotide used for screening (3NTU) was derived from the presumed 3' untranslated region of the nidogen mRNA (21). This sequence is no longer present in the final cDNA sequence of nidogen (35, 36) and therefore may be a cloning artefact. Nevertheless, the oligonucleotide hybridized in Northern blots to two RNAs of about 4.8 and 6 kb as was expected for nidogen mRNAs and, in addition, weakly to several larger RNAs. Screening of the library PG3 revealed about a dozen positive signals. Clones belonging to the strongest signals were grown up and plasmid DNA was analyzed by restriction enzyme digestion. Three plasmids, from clones N1, N14 and N29, were characterized in detail. Fragments for subcloning and sequencing were isolated from all clones, but only the 5' and 3' ends were sequenced for each of them. The Sall fragment of plasmid N29 was used to screen library cII (20) to isolate longer inserts. This screen resulted in clone NN10.

A Northern hybridization with plasmid N1 revealed a series of bands between 13 and about 2 kb which could not belong to (33). The genomic DNA was digested with different restriction enzymes and separated on 0.8% agarose gels. The denatured gels were dried down onto filter paper (cf., 22, 34) and used directly for hybridizations in order to obtain a higher sensitivity. The gels were hybridized under the same conditions as described for Northern blots with synthetic oligonucleotides and washed as indicated.

PCR analysis
Genomic DNA (120 μg) from PYS-2/12 cells was digested to completion with Sall. The DNA fragments were separated on a 1% low melting agarose gel (Merck). The gel was cut into slices and the DNA recovered by electroelution. Part of a fraction with a size of about 1.5 to 1.8 kb was diluted 1:10 and used as template (1μl) for PCR reactions.

As primers the oligonucleotides RL18S (5' AAGCTCGTATGGTGCGCTGGTAG 3', complementary to L18S) and Spfl8 (5' CTAACCGTATGCGATCCAGAGT 3' ) comprising the Spfl site close to the end of 18S were used. The reactions were performed in a Perkin-Elmer thermocycler 480 with vent polymerase (New England Biolabs)) and an annealing temperature of 66°C. The amplified product of about 1 kb was purified on a 4% polyacrylamide gel and ligated to pUC830 cut with EcoRV. Four positive clones were sequenced.

Fig. 1. Schematic representation and restriction map of the cloned sequences. The shaded boxes represent the 18S RNA, the black box the B2 element. The stippled box indicates the part of 18S RNA obtained by PCR. The combined sequences derived from four cDNA clones are shown at the bottom by a thick line, the polylinker of the vector is shown as thin lines. Some of the restriction sites used for fragment isolation and subcloning are marked by thin vertical lines (A, Ava; B, BamHI; D, DraI; E, EcoRI; H, HindIII; K, Kpnl; P, PstI; S, Sall; Sc, Sacl; Sp, Sphl; X, XbaI). The 5' ends of the four clones are indicated by arrows. The thick line on the right hand site depicts the genomic fragment amplified by PCR.

Fig. 2. Sequence of the B2 element and part of the 5' and 3' adjacent 18S sequence. The 3' adjacent sequence (poly(A) tail and direct repeats in bold) was obtained from the cloned PCR fragments. The following 18S sequence has been omitted. The genomic DNA was digested with different restriction enzymes and separated on 0.8% agarose gels. The denatured gels were dried down onto filter paper (cf., 22, 34) and used directly for hybridizations in order to obtain a higher sensitivity. The gels were hybridized under the same conditions as described for Northern blots with synthetic oligonucleotides and washed as indicated.
nidogen mRNAs. As the pattern strongly resembled bands which were seen in ethidium bromide stained RNA of PYS cells separated on methylmercury gels (not shown), I investigated these cDNA clones further.

**Characterization of the cloned fragments**

Fig. 1 gives a restriction map of the combined DNA sequence obtained from clones N1, N14, N29 and NN10 and a schematic representation of the position relative to the ribosomal precursor. The 3' end of the cloned sequences (Fig. 2) consists of a B2 element (37), a retroposon characteristic of rodent genomes. The B2 element is about 94% identical to the general consensus sequence and nearly 97% identical to the consensus sequence of type II B2 elements (14). Cloning of the 3' adjacent sequence by PCR showed that the B2 element is surrounded by a perfect direct repeat of 15 bp which begins with five consecutive A residues, in agreement with insertion of retroposons into A-rich sequences (14—16). The tail contains only A residues and is at least 50 nt long, its length varied slightly for four independent clones. The unusual length of the poly (A) tail may explain why the RNA from which the clones are derived is present in large amounts in oligo(dT)-selected RNA (cf., Fig. 4).

The upstream sequences are nearly identical to part of the 5' ETS and the 5' end of the 18S rRNA. The differences in our rDNA sequence (EMBL Data Library, accession no. X56974) with respect to the recently published murine rDNA sequence (10) could be sequencing artefacts in the published data or another example that not all rDNA transcription units are identical (cf., 13). The sequence also revealed four mismatches with respect to oligonucleotide 3NIU used for screening.

The nucleotides 3' adjacent to the B2 element obtained from cloned PCR products are included in Fig. 2 (cf., Fig. 3). The sequence further 3' does not differ from the 18S sequence for at least 200 nt. Also the sequence from the 3' end of the PCR fragment matches 18S sequence for at least 400 nt (one mismatch). The residual sequence of the PCR fragment was read with several mismatches which could be due to compression of bands or errors from PCR. Therefore I conclude that the B2 element has been inserted into a normal 18S rRNA unit.

**Northern hybridizations**

As the cDNA libraries were constructed from poly(A)-enriched RNA equal in length or larger than 28S RNA by annealing poly(A) tails to a poly(T) tailed vector (20), the clones cannot be full-length transcripts from the parent RNA(s). In order to identify the (se) template(s), Northern blots were hybridized with different portions of the cDNA clones and with oligonucleotide L18S.

After hybridization with oligonucleotide L18S, four RNAs of about 13, 6.1, 4.4 and 1.9 kb were detected (Fig. 4A) in PYS cells. The same bands, within smear all along the lanes, were seen with the PstI-Dral fragment (B2 element) subcloned in M13 (Fig. 4B, 5B), albeit with differing intensities. Fig. 4C shows that the three largest RNAs are enriched in poly(A)+ RNA compared to total RNA while the 1.9 kb RNA is missing. Only the 1.9 kb band is seen in RNA from cell lines other than PYS (Fig. 4). Oligonucleotide L18S detected a similar RNA also in rat and human RNA (not shown). Staining of blots with aurintricarboxylic acid (27) prior to hybridization revealed comigration of the 1.9 kb band with 18S rRNA. The oligonucleotide hybridized even to RNA of the size of the small ribosomal RNA from E. coli, yeast and dictyostelium (not shown) so that it is most likely that the 1.9 kb band results from weak hybridization to the abundant 18S rRNA, as L18S is complementary to this sequence for 14 nt. The M13 clone with the B2 element (Pst-I-Dral) also contains 25 nt complementary to 18S rRNA and may therefore stick to it.

![Fig. 4. A: Northern blot hybridized with oligonucleotide L18S in parallel with the Southern gel shown in Fig. 7C. Both were washed together under the same conditions (59°C). The different lanes contain 4 µg each of total RNA from PYS-2/12 (a), HR9 (b) GZ (c) and C1300 (d) cells. B: Northern blot with total RNA from PYS-2/1 (a), PYS-2/3 (b), PYS-2/11 (c) and HR9 cells (d). The blot was hybridized with the Pst-Dral fragment containing the B2 element (subcloned in M13). C: Northern blot with total RNA (4 µg) of mouse embryo fibroblasts (a) and PYS-2/12 cells (b) and poly(A)+ RNA (1µg) of PYS-2/12 (c) hybridized with oligonucleotide L18S. The blot was hybridized and washed at 43°C in 0.2 x SSC and 1% sarcosyl and exposed for 3 days. After washing at 56°C in 3.2 M tetramethylammonium chloride (TMACl) (42), only the main band (6.1 kb) in poly(A)+ RNA (d) remained strong enough to be detected after 6 days.](image-url)
Fig. 5. Decay of ribosomal RNA precursors. Actinomycin D (80 ng/ml (42)) was added to HR9 and PYS-2/12 cells which were harvested after 0 (1), 30 (2), 60 (3), 120 (4) and 240 (5) min incubation for preparation of total RNA. 4 ug of total RNA from the different time points were electrophoresed on a 0.7% agarose gel. Lanes 6 contained 4 ug of cytoplasmic RNA. The Northern blot was hybridized successively with the Sacl-Sphl fragment (subcloned in Ml3) from the 5' ETS (A) and oligonucleotide L18S (B). The blot in (B) was washed in 3M TMACl at 60°C.

An RNA of about 2 kb has been detected in (38) when probing Northern blots with a cloned B2 element. As L18S might also crosshybridize to that RNA, an oligonucleotide analogous to L18S, containing at the 5' end the sequence (cf., Fig. 2) given in (38), was hybridized to Northern blots. No signal was obtained.

The 13 kb band corresponds in size to the ribosomal primary transcript and/or the first processing products (cf. Fig. 6A). The 6.1 kb band could represent an rRNA similar to 34S RNA which is 6.2 kb long. As the 6.1 kb RNA also has to contain the B2 element of about 250 nt, either the 5' ETS and ITS 1 have to be shorter or ITS 1 might be missing and the complete 5' ETS may be present. The 4.4 kb band could be derived from the 6.1 kb RNA by further shortening of the spacer portions or could contain the complete 5' sequence up to the end of the B2 element. The proposed RNA species with the retroposon are depicted in Fig. 6B.

Fig. 5A shows a Northern blot hybridized with the Sacl-Sphl fragment (cf., Fig. 1) detecting all rRNAs containing the 3' end of the 5' ETS (lanes 1). The most prominent bands are about 13 and 6.2 kb, corresponding to the primary rRNA transcript (47S-45S) and 34S precursor which are not present in cytoplasmic RNA (lanes 6). After incubation with actinomycin D at low levels, supposed to inhibit mainly transcription of rRNA (42), these precursors vanish within 120 min. The 13 kb precursor disappears even quicker in RNA from PYS-2/12 cells, while the 6.1 kb band persists somewhat longer than in HR9 cells.

When the same blot is rehybridized with the B2 element (Fig. 5B), only the 1.9 kb band comigrating with 18S RNA is seen in RNA from HR9 cells, while RNA from PYS-2/12 cells contains a faint band of about 13 kb (arrow) and a strong band of 6.1 kb in addition to the 1.9 kb band. The 6.1 kb band decays rather slowly and no additional bands which might result from further processing appear. This implies that the 6.1 kb RNA cannot be processed at the 5' end of 18S RNA (cf., Fig. 6B) and may be slowly degraded. Therefore the 18S rDNA with the inserted retroposon can be regarded as a transcribed pseudogene.

Fig. 6. Schematic representation of rRNA precursor fragments. A: Representation of the fragments according to (3). B: Representation of the putative RNAs containing the B2 element (black box). Their structure is deduced from the cDNA sequences, the hybridizations in Fig. 4 and 5 and similar blots hybridized with different parts of the cDNA clones (not shown). The depicted RNAs are compatible with the length of fragments seen in Northern blots. The broken lines indicate that variable portions of the 5' and 3' spacer segments could be present in molecules of the same length.

Analysis of genomic DNA
The insertion of the B2 element and the relatively large amount of RNA with sequences of the 5' ETS prompted an analysis of genomic DNA of several of our mouse cell lines. The lengths of fragments containing the B2 element for several restriction enzymes was predicted by adding about 200 bp to the fragments depicted in Fig. 7A. The synthetic oligonucleotide L18S (complementary to the junction between 18S and B2 element) was hybridized to dried gels containing genomic DNA digested with restriction enzymes which cut close to the integration site of the B2 element (cf. Fig. 7A). In the Xbal and Smal digests of DNA from PYS-2/12 cells, there are fragments which are 260 and 245 bp longer than expected (cf.; Fig. 7A) for the ribosomal gene unit. This is in perfect agreement with the insertion of the B2 element (245 -250 nt, depending on the actual length of the poly(A) tail). The normal, smaller fragment without the B2 element does not hybridize under the hybridization and washing
conditions, in contrast to the results from Northern blots where oligonucleotide L18S also bound weakly to 18S RNA (cf., Fig. 5). The Sall and BamHI digests contain a fragment that is about 500 bp larger. This size difference cannot be explained from the present data and may indicate additional changes within the sequence of the ITS for this special rDNA unit. The bands in the other digests have roughly the expected positions, but they are too large to allow a precise measurement.

All these bands are not present in embryonic mouse DNA which was derived from a C129 mouse (Fig. 7B, 8). For a comparative digest of DNA from different cell lines, I chose BamHI as the larger bands were retained much better in the gels and gave stronger hybridization signals. The gel in Fig. 8 reveals that only our different PYS sublines contain the B2 element. Even those cell lines (HR9, F9) derived from the same teratocarcinoma (OTT6050) as the PYS cells are devoid of it (cf., 22). This result indicates to me that the B2 element must have been inserted into a ribosomal gene unit of either a subline of tumor OTT6050 or the PYS cells during 1970 to 1974 (see Discussion, cf., 22). The gels do not allow to determine the copy number of 18S rDNA with the B2 element. As oligonucleotide L18S only detected the specific bands in dried gels which are supposed to give about five times stronger signals with oligonucleotides compared to blots (39) and as we had to expose the gels for a week, this would fit with a single copy. On the other hand, the signal obtained from the DNA of different cell lines varies. This could be due, in part, to somewhat different amounts of DNA loaded, but judged from the staining of the gel, lane 1 (Fig. 8) contained more DNA than lane 3 which gives the stronger signal. Therefore it seems possible that there could be up to four copies...
of this rDNA unit or that there are mutations in the sequence matching the oligonucleotide in different cell lines.

All DNAs contain a crosshybridizing fragment which must be derived from a multicopy gene. When the Southern were washed at 50°C, this was the only fragment detected with oligonucleotide L18S after short exposures. Washing at 55°C or 60°C decreased the intensity of this band until it had roughly the same intensity as the specific, expected fragment (cf., Fig. 7, 8). A search of the MIPSY database ( Martinsried) with the sequence complementary to oligonucleotide L18S did not identify any possibly crosshybridizing sequence.

The signal intensity for the crosshybridizing band also varies in such a way that it does not agree with the differences in DNA loading. This could either be explained by different copy numbers of the crosshybridizing DNA or a different degree of divergence from the oligonucleotide sequence.

DISCUSSION

The cDNA clones presented are derived from a prominent RNA from our PYS cells. The 13 and 6.1 kb bands seen in stained RNA gels (not shown) were always a characteristic feature of our RNA preparations. Cloning of part of these RNAs established them as ribosomal RNA precursors. In addition, the cDNA clones revealed a transcribed pseudogene of 18S rRNA containing a retroposon (B2 element).

Origin and properties of the B2 element

All cell lines used for the Southern analysis except C1300 cells were derived from the tumour OTT6050 (42) which stems from a teratocarcinoma of a strain 129 mouse. In 1973 several sublines of tumor OTT6050 are mentioned in (43). It is not clear from the literature which subline of the tumor was used to establish the PYS cells and the precursors of the HR9 cells, e.g. whether they were derived from the same subline as the F9 cells (OTT6050–970) or whether they stem from different tumor sublines. In any case, the publishing dates for the different cell lines (cf., 22) delimitate the time for the event of insertion of the B2 element to 1970 through 1974.

As B2 elements are especially frequently expressed in embryonic cells or teratocarcinoma-derived cell lines or brain tissue (14, 16, 44), the integration of the B2 element presented here could have been facilitated either in the teratocarcinoma subtype or the progenitors of the PYS cells. The insertion of a B2 element may even have happened again in the subline PYS-2/11 (cf., Fig. 7B) where an additional, larger fragment hybridized to the probe. The high degree of identity of the cloned B2 element with the consensus sequence (97%) also points to a recent transposition event. This may have been facilitated by the exceptionally long poly(A) tail. It is not known whether transcripts of B2 elements are polyadenylated similar to mRNAs (they contain the polyadenylation signal AATAAA ( Fig. 2, 3)) or whether the tail is already encoded in the primary transcript.

Impact of the B2 element on transcription and processing of the mutated rRNA unit

Retroposons have already been detected in the rDNA intergenic spacers of e.g. rat and human (45, 46), but only for insects, retroposons within ribosomal RNA (28S RNA) have been described (47 and references therein; 48). These repetitive elements are 4.2 or 5.1 kb and may code for proteins relevant to their insertion (47). Those elements seem to repress transcription of ribosomal RNA which is definitely not the case for the B2 element presented here. Southern hybridization (Fig. 8) suggests that the 18S gene with the retroposon is a single copy gene present in only few copies which means an excess of about 200 normal rDNA copies. The Northern hybridizations in Fig. 4 and 5 reveal that a substantial portion of the 13 kb precursor contains the B2 element. The partially processed precursor (6.1 kb band) still accumulates after blocking of transcription by actinomycin D (Fig. 5). These results may even be compatible with an enhanced transcription of this particular gene unit. An Alu element has been shown to function as enhancer for a gene unit transcribed by RNA polymerase II (50) but it is unknown whether similar effects are possible for transcripts of RNA polymerase I, e.g., the ribosomal genes.

In Fig. 5B, the 6.1 kb band disappears without the appearance of strong other bands, but may be slightly shortened during the incubation period, pointing to inhibition of processing at the 5' end of 18S RNA. According to the accepted structure of mammalian 18S RNA (51) the B2 element would be inserted just behind stem 21. The B2 element itself can be folded into a highly base-paired structure and would then be part of the variable region V4. According to the proposal of (52) for an alternate stem 21, the point of insertion would be in the small loop between stem 21a and 21b, or, as stem 21a contains the eleven last nt of the direct repeat surrounding the B2 element, the insertion could also be placed into the unpaired region between stems 20 and 21. Despite of the possible folding of the B2 element, such an insertion presumably would prevent formation of stem 20 and/or 21 because of about 80 nt (poly(A) tail and 5' adjacent A-rich region) of unpaired sequence. As this region is close to the beginning of 18S rRNA in the present model of secondary structure (51), this may offer an explanation why the ribosomal precursor with the B2 element (6.1 kb) cannot be processed at the 5' end of 18S rRNA.
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REFERENCES


