Integration of a vector containing rodent repetitive elements in the rat genome

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ABSTRACT
We have previously shown that integration of a polyoma vector containing rodent repetitive elements into rat cellular DNA is non-random (Wallenburg et al. J. Virol. 50: 678–683). Junctions between the polyoma vector and the host DNA occur in the repetitive sequences of the vector about ten times more frequently than would be expected if sequences from the vector were used randomly for integration. In this paper we looked at the host sequences involved in these junctions. Our analysis did not reveal any repetitive or specific sequences and we presume therefore that the repetitive sequences of the vector acted as hot spots for illegitimate recombination. We also analysed the integration mechanism and found that: First, even though the polyoma vector was transfected in the presence of carrier DNA, integration did not involve the formation of a transgenome. Second, in at least one of the clones analysed, integration resulted in deletion of host DNA sequences. Third, the host DNA displaced at the integration site was considerably longer than the integrated segment.

INTRODUCTION
Highly repeated short and long interspersed sequences (SINES and LINES) appear to be an ubiquitous component of mammalian genomes (1, 2, 3). Amongst the better characterized are the Alu (4) and KpnI (5) repetitive families in human, and Bl (6), B2 (7) and MIF-1 (8) repetitive families in mouse. These elements have copy numbers of about 10⁵ that are scattered through their host genome. The role, if any, of these repetitive units remains unknown. They would appear however, to be capable of various recombination processes such as retroposition, gene conversion, translocation, excision, homologous and non-homologous recombination (reviewed in 2, 3).

We previously investigated the integration in the rat genome of a polyoma derived vector (RmI) containing rodent repetitive sequences (9). RmI (10, 11, Fig. 1) consists of a complete polyoma genome with an insertion of mouse cellular sequences (Ins), that contains copies of two rodent repetitive elements, B2 (7) and MT (12). We observed that integration of RmI in rat cells occurred preferentially via the repetitive sequences present in Ins. These results were subsequently confirmed in a separate study.
In fact, junctions between host cellular DNA and Rml occurred about ten times more frequently in the portion of Ins containing the repetitive sequences than would have been expected for a random process. To find out more about the recombination hot spot behaviour of the repetitive sequences of Rml, we cloned and sequenced four Rml-cellular junctions occurring in the repetitive sequences of Rml. The detailed analyses of these junctions are presented in this paper. We could not identify host sequences at the junctions that were repetitive or specific and presume therefore that the repetitive sequences of Rml acted as hot spots for illegitimate recombination events.

In studying the integration mechanism, we also made the following observations: first, even though Rml was transfected into rat cells with carrier DNA, it integrated directly into the host genome and not via the formation of a transgenome (14, 15, 16). Second, in at least one of the clones analysed, integration was accompanied by deletion of host DNA sequences and finally the host DNA sequences displaced at the site of integration were considerably longer than the ones being inserted.

**MATERIALS AND METHODS**

**Cell lines**

Cell lines W98.12 and W98.14 have been described previously (9). They were produced by transfecting Fisher rat 3T3 (FR3T3) cells (17) with Rml using the calcium phosphate technique (18, 19). The carrier DNA used was high molecular weight DNA isolated from mouse LTA cells (19). Clone 11.6 was produced by using the same protocol with minor modifications (56). Briefly, 5 x 10^5 FR3T3 cells were seeded on a 90 mm petri dish 24 hours prior to transfection with 0.4 micrograms Rml and 13 micrograms of salmon sperm carrier DNA. The medium was replaced with fresh medium 20 hours post transfection, and 44 hours post transfection, cells were trypsinized and reseeded at 5 x 10^4 cells/60 mm petri dish. After 14 to 17 days, foci of densely growing cells were isolated, subcloned and grown up for DNA extraction. Clone 11.6 contains a single insertion of Rml as determined by detailed restriction enzyme analysis (56).

**DNA preparation and restriction enzyme analysis**

Total cellular DNA was prepared as previously described (9). Restriction enzymes were used as suggested by the manufacturer with a 5x excess of enzyme. For analyses of cellular DNA, 5 to 10 micrograms of high molecular weight DNA were digested. Rml was prepared as previously described (10).
The physical structure of Rml as previously determined (9, 10). The numbers refer to standard polyoma map units and OR identifies the polyoma origin of replication. The early and late coding regions have been indicated with the late region interrupted by Ins. Ins is an insertion of mouse cellular DNA. B2 and MT refer to the B2 (11) and MT (12) families of rodent repetitive DNA.

Probes and hybridization

For Southern analyses the DNA was transferred to nitrocellulose (type BA85, Schleicher & Schwell Inc.) or nylon (Hybond, Amersham) membranes by the technique of Southern (20), with the modifications suggested by the manufacturer. The hybridization procedure was that of van der Ploeg and Flavell (21) with minor modifications (22). Probes were prepared by the nick translation procedure of Rigby et al (23) or by using the random hexamer-primed synthesis technique (24).

Cloning the Rml-cellular DNA junctions

High molecular weight cellular DNA was digested with EcoRI and the lengths of the Rml-cellular DNA junctions determined. The appropriate fragments were enriched on sucrose gradients (25) and recovered by two rounds of ethanol precipitation. The cloning was done in vectors λgtWES, λB (26) or λL47.1 (27) followed by in vitro packaging. The junction-containing recombinants were identified by plaque hybridization (28) against a polyoma probe, and the inserts subcloned into pBR322 at the unique EcoRI site, except 11.6L which was cloned in pAT153/EcoRI. The initial site of 11.6 (IS) was cloned by a similar procedure as a 9 Kbp BglII fragment into λL47.1 digested with BamHI. An EcoRI subfragment of this recombinant, a fragment of 2.6 Kbp, was subcloned into pBR322.
Figure 2

The physical maps of the cloned Rml-cellular junctions. Each junction was cloned as an EcoRI fragment as described in Materials and Methods. Thus both EcoRI junctions of a single clone are shown in juxtaposition separated by the EcoRI site of Rml. The name of the clone from which they were derived is given on the extreme left. The inserted sequences of Rml have been aligned with the linearized representation of Rml given at the top of the figure. (Ins has been duplicated for the purpose of illustration.) The legend for the Rml sequences is the same as in figure 1. The line represents the cellular sequences flanking each insertion. IS is an EcoRI cellular fragment that represents the arrangement of the cellular sequences at the left side of the Rml insertion in clone 11.6 before integration. It has been aligned with the corresponding cellular sequences in 11.6 directly above. S1 through S7 delineate the probes used for the restriction enzyme mapping of the initial sites. S8 is the probe used to determine the fate of the initial sequences after integration (see figure 6A). Restriction enzymes: A, HpaI; B, BamHI; E, EcoRI; F, Hinfl; G, BglII; H, HindIII; I, BglI; J, Aval; K, KpnI; M, SmaI; F, PstI; S, SalI; V, PvuII; X, XbaI. Not all restriction sites are shown. Sites with an asterisk were used to sequence the junctions.

Sequencing

The sequences across the junctions were determined using the chemical cleavage procedure of Maxam and Gilbert (29, 30).

Sequence comparisons

The determined sequences were compared to published sequences for the Bl (6), B2 (7), R (31) and MT (12) consensus sequences and Bam5 (32), and L1Rn (33) families of repetitive DNA, using the DOTMATRIX (stringency=60, 70 or 80% over 10 bases) and HOMOLOGY (stringency=65% over 20 bases) programs of Stephens (34).

Copy number

Relative copy number of DNA fragments from Southern analysis were
Cloning of the Rml insertions in clones W98.12, W98.14 and 11.6

Clones W98.12, W98.14 and 11.6 were selected for analysis of the role played by repetitive sequences in the integration of Rml in the rat cellular genome. Each of these clones contained only one Rml insertion and in each case at least one Rml-cellular DNA junction had occurred in the repetitive sequences present in Rml (Fig. 2). Restriction enzyme mapping located the right junction of insertion W98.12 (12R) and the left junction of insertion W98.14 (14L) in the B2 repetitive sequences present in Rml, while the left junction of W98.12 (12L) and the left junction of 11.6 (11.6L) mapped in the HT repetitive sequences present in Rml. Total cellular DNA from each clone was digested with EcoRI and each junction cloned separately (see Material and Methods).

Nature of the endogenous nucleotide sequences at the junctions

The restriction sites designated by asterisks in Fig. 2 were used to determine the nucleotide sequences of Rml-cellular junctions 12R, 12L, 14L and 11.6L (Fig. 3). These have been aligned with the corresponding Rml determined by densitometry scanning of autoradiograms using an LKB Utlroscan XL and accompanying Gelscan XL software.

RESULTS

Cloning of the Rml insertions in clones W98.12, W98.14 and 11.6

The sequences across the junctions shown in comparison to the corresponding sequences of Rml. 11.6L is also compared to the initial site (IS). Arrow heads indicate the cross-over point in each junction. Vertical lines are drawn between homologous bases. Dashes have been inserted to allow alignment of homologous sequences. Bases with a halo (in the sequence of 12-R) are homologous with L1Rn (see text).
**Figure 4**

Rml integrated directly into host cellular DNA and not into the carrier DNA. Five micrograms of clone (W or 11), host FR3T3 (F), and carrier LTA (L) and 5 (S) or 10 micrograms (S') of carrier salmon sperm. DNAs were digested with restriction enzymes, migrated beside each other on agarose gels, blotted and hybridized to probes of cellular DNA. The probes (S2, S3, S4 and S5 shown in Fig. 2), were chosen to contain only unique sequence DNA and to cover the region immediately flanking the site of integration. Probe S7 (Fig. 2) hybridized to sequences present in the order of 5 to 10 copies per genome. The junctions analysed and the probes used are indicated beneath each autoradiogram. Restriction enzymes: G, BglII; H, HindIII; P, PstI; X, XbaI. The host DNA immediately flanking 11.6R (which includes the Xba site) has undergone a rearrangement, and therefore this Xba fragment (lower band in 11.6) does not comigrate with the unrearranged fragments (upper band in 11.6 and FR3T3). Note that the single copy rearranged band is several times less intense than the unrearranged bands.

sequences. The junctions are homologous with Rml up to a certain point (see arrowhead in Fig. 3) beyond which there is no significant homology. In all four cases the break point occurred in repetitive sequences of Rml, either B2 (12R, 14L) or MT (12L, 11.6L). The preferential involvement of Rml repetitive sequences in integration could have been the result of homologous recombination with endogenous repetitive sequences of the host genome. However, if this had been the case, we would have expected to find at the junctions, complete chimaeric repetitive elements originating in part from Rml and in part from the host. What we found at the junctions in all cases were truncated repetitive elements linked to sequences unrelated to the repetitive element. There is some divergence between the repetitive sequences at the junctions and the repetitive sequences present in Rml. We do not know how these originated, but it should be noted that Rml is
produced by amplification in mouse cells and these base differences could represent variations between Rml molecules. They could also be mutations induced by transfection or by integration (35, 36). To confirm that homologous endogenous repetitive sequences were not present at the junctions, we used the flanking cellular sequences of junction 11.6L (Fig. 2, fragment S5) as a probe to clone the cellular sequences of 11.6L in their original arrangement before integration. This segment is designated IS in Fig. 2. We determined the nucleotide sequence across the site where Rml had integrated. (IS in Fig. 3). This sequence was found to be homologous to the cellular sequence found at the 11.6L junction but had no significant homology with the repetitive sequences of Rml present at the junction. Thus junction 11.6L occurred in repetitive sequences of Rml but did not involve homologous repetitive sequences of the rat cellular genome. Although as mentioned above, the structures for junctions 12R, 12L and 14L make it highly unlikely that homologous cellular sequences were involved in integration, we cannot exclude this possibility since the nature of the original host sequences was not determined.

It has been proposed (37) that integration of exogenous DNA occurs preferentially in host cellular repetitive sequences. We did a computer search of the sequences for the presence of highly repetitive sequences in the cellular host DNA at or near the junctions (see Material and Methods). None of the four cellular junctions had significant homologies with the major families of rodent repetitive sequences, including B1 (6), B2 (10), R (31), Bam5 (32), MT (12) or L1Rn (33), with the exception of a sequence close to junction 12R (Fig. 3). This sequence had a homology of 78% over 56 bp, including a 36 bp stretch of 90% homology, with a middle segment of the LINE repetitive element L1Rn (33). Furthermore, probes derived from the cellular DNA at (Fig. 2, fragment S3) or near the junctions (Fig. 2, fragments S2, S4, S5, S6, S7, S8) hybridized with only one band of host DNA (see Fig. 4 and 6). Probes S6 and S7 hybridized with sequences present in the order of 5 to 10 copies per genome (Fig. 4). Thus our results do not indicate that integration would occur preferentially in host highly repetitive DNA. Finally, in comparing the host sequences at the junction, we did not find any common sequence or structure at or near the junction.

**Analysis of the integration mechanism**

It has been shown that when DNA is transfected into mammalian cells with carrier DNA, it can form structures designated transgenomes or pekelosomes that can integrate into the host DNA (14, 15, 16). Transgenomes or pekelosomes are high molecular weight DNA structures of several hundred Kbp that result from ligation between the transfected DNA and the carrier...
DNA. Since we wished to examine the interaction between Rml and endogenous host sequences, it was necessary to determine whether Rml had integrated directly into the host DNA or via a transgenome. As we had used salmon sperm or mouse DNA as carrier, cellular DNA segments near the junctions were used as probes in Southern analysis to determine if the DNA flanking the Rml insertions originated from the host or carrier DNA (Fig. 4). For each junction we hybridized the corresponding cellular probes (S2, S3, S4, S5, S7 in Fig. 2) to DNAs originating from the clones (lanes W or 11), from the parental FR3T3 cell line (lanes F) and from the carrier mouse LTA (lanes L) or salmon sperm (lanes S and S'). The left junction of clone 98.12 (12L) could not be analysed because too few non-Rml sequences had been cloned to make a probe (Fig. 2). The restriction enzymes used to digest the DNA introduced a cut in the cellular sequences near the cross-over point (see Fig. 2). Thus if the cellular sequences at the junction originated from the host, we would expect the probe to hybridize with bands co-migrating in the clone and the parental cell line and not present in the carrier. This is what we found in all cases (Fig. 4). With clones 98.12 and 11.6 there was no hybridization between the probes and the carrier. Probes S3 and S4 (from clone 98.14) did hybridize weakly to non co-migrating bands in the carrier DNA. We believe this was due to cross-hybridization between rat and mouse DNA. For junctions 12R and 14R we see more than one band in both the clones and the parental line. This is because the probe contains a site for the restriction enzyme used in the analysis and thus the probe hybridizes to more than one fragment of cellular DNA. We repeated such analyses with a number of restriction enzymes (data not shown) and the results confirmed that the flanking cellular sequences of all the junctions analysed originated from the parental FR3T3 DNA and that carrier was not involved in the integration of Rml. Furthermore in the case of junction 11.6L we have already shown that the nucleotide sequences up to the Rml insert originated from the parental FR3T3 genome (Fig. 3). Thus we conclude that integration of Rml did not involve the formation of transgenomes or pekelosomes, even though Rml was transfected in the presence of carrier DNA.

Rearrangement of host sequences at the site of integration of Rml

The cellular probes described above, S2, S3, S4, S5, S6 and S7 could be used to deduce the physical map of the host sequences contiguous to the integration sites before integration. This was done by establishing their restriction maps in the parental cell line. These maps are presented in Fig. 5 along with the physical map of the flanking host sequences at the integration sites. In none of the three clones were we able to establish that the host sequences flanking each side of the integration site were
Maps of the rearrangements of the cellular DNA associated with the integration of Rml. Beside the name of each clone (given on the extreme left) is the map of the cellular DNA after integration. (The integrated sequences of Rml are represented by wavy lines.) Aligned above and below each map are the maps of the initial sites before integration as determined by Southern analysis using probes of cellular DNA, shown as solid bars. These correspond to probes S2 to S7 in figure 2. Open bars: cellular DNA flanking the insertions. Solid lines: cellular DNA before integration. Hatched bars: cellular DNA flanking the insertion in 11.6 which has been reorganized (see text). -R-: initial site as mapped by a probe from the right flanking sequences. -L-: initial site as mapped by a probe from the left flanking sequences. Restriction enzymes: A, HpaI; B, BamHI; E, EcoRI; G, BglII; H, HindIII; I, BglII; K, KpnI; M, SmaI; P, PstI; V, PvuII; X, XbaI. Not all restriction enzyme sites are shown.

Figure 5

Maps of the rearrangements of the cellular DNA associated with the integration of Rml. Beside the name of each clone (given on the extreme left) is the map of the cellular DNA after integration. (The integrated sequences of Rml are represented by wavy lines.) Aligned above and below each map are the maps of the initial sites before integration as determined by Southern analysis using probes of cellular DNA, shown as solid bars. These correspond to probes S2 to S7 in figure 2. Open bars: cellular DNA flanking the insertions. Solid lines: cellular DNA before integration. Hatched bars: cellular DNA flanking the insertion in 11.6 which has been reorganized (see text). -R-: initial site as mapped by a probe from the right flanking sequences. -L-: initial site as mapped by a probe from the left flanking sequences. Restriction enzymes: A, HpaI; B, BamHI; E, EcoRI; G, BglII; H, HindIII; I, BglII; K, KpnI; M, SmaI; P, PstI; V, PvuII; X, XbaI. Not all restriction enzyme sites are shown.

linked before the integration event. That is, in none of the three cases did we find a host fragment that would hybridize with probes from both sides of the integration site nor could we match the restriction maps established with the probe from one side with the restriction maps established for the other side. What we can deduce from these maps however, is that before integration the flanking cellular sequences of clones W98.12, W98.14 and 11.6 were separated by at least 12 Kbp, 55 Kbp and 90 Kbp respectively. These numbers were obtained as follows: in the case of clone W98.12 we could establish the restriction map of the cellular site before integration up to 12 Kbp to the left of the S2 probe and it differed totally from the...
restriction map of the cellular sequences present at the left of the integration site (see Fig. 5). In the case of clone W98.14, before integration we found that the restriction map for at least 29 Kbp to the right of the S3 fragment and 26 Kbp to the left of the S4 fragment were distinct. Thus S3 and S4 were originally separated by at least 55 Kbp. Finally in the case of clone 11.6, we found that digestion of the parental DNA with BgIII generated a fragment that in low concentration agarose (.35%) migrated much higher in the gels than 50 kb. We estimate that this fragment extends 90 Kbp to the right of S5. It did not hybridize with either S7 or S6. We also found that the cellular DNA immediately flanking the 11.6R junction (Fig. 5, hatched region) had undergone rearrangement as compared to the host sequences from the right hand side before integration (see Fig. 4 legend). However, using the S6 and S7 probes (Fig. 2) we could establish that these rearranged flanking sequences were linked with the unrearranged distal cellular sequences before integration but in a different configuration (data not shown).

The above results raised the question: were the host flanking sequences linked before integration or did they originate from distinct chromosomes? A fortuitous event indicates that at least in clone W98.14 the flanking cellular sequences appeared to be linked together before integration. This is illustrated in Fig. 6A. We have put side by side DNA from W98.14 and FR3T3 and hybridized them with probes S3, S4 and S5. The relative intensity of the resulting bands was determined by densitometry scanning (see Material and Methods). S5 hybridizes to a cellular band unrelated to the integration site in clone W98.14 and it was used to correct for small variations of intensity due to variation of DNA content in each lane. When the band intensities are corrected, we find that the S4 bands and the total of the S3 bands are twice as intense in the W98.14 clone than in the parental FR3T3. Thus there are twice as many copies of the flanking cellular sequences in the W98.14 clone than in its parental clone. But when we look at the integration site as illustrated by the lowest S3 band for which there is no corresponding band in FR3T3, we find it to be only half as intense as the upper S3 band in FR3T3. This tells us that the amplified copies are the allelic sequences and not the integration site. This amplification of the flanking sequences is also evident in Fig. 4. A simple explanation for these results is that integration occurred fortuitously in sequences that were amplified compared to the parental sequences. The fact that the sequences from both sides of the integration site have been amplified to an equal extent in an event unlinked to integration, suggests that these sequences were linked prior to integration. Note that the amplification did not affect
unrelated sequences as judged by the S5 bands that are of equal intensities in both lanes.

Did integration result in deletion of host sequences? To answer this question we digested 11.6 and FR3T3 DNA with PstI, which separates the flanking unrearranged host DNA from the host DNA rearranged by the integration of Rmi (Fig. 2, 11.6 and I.S.). We then hybridized with the S5 probe homologous to the unrearranged host DNA and the S8 probe homologous to the rearranged host DNA. The results are shown in Fig. 6B. We did a densitometric scanning of the autoradiogram and used the S5 band to correct for variation of DNA content in each lane. After correction, we find that the band hybridizing to the S8 probe is only half as intense in 11.6 as in FR3T3. Since no other band hybridizing to the S8 probe is found in 11.6 DNA, we conclude that the rearranged host DNA has been deleted. This was confirmed by analyses with other restriction enzymes (data not shown).

**DISCUSSION**

We had previously established that exogenous repetitive sequences were hot spots for integration in the rat genome (9, 13). In this paper, we examined the host DNA into which the exogenous DNA had integrated. Our analysis failed to reveal the presence of repetitive or specific host sequences. Thus it would appear that integration occurred through
illegitimate recombination and that repetitive sequences can act as hot spots for such events. This would explain, as has been hypothesized by Rogers (38), why repetitive sequences are often found at illegitimate junctions such as translocation break points (39, 40, 41) or in cellular excision product referred to as small polydispersed circular DNA (spc) (42, 43, 44). It also raises the interesting possibility that if reintegration of spc DNA in the host genome was to occur it would preferentially occur via the repetitive sequences. This would preserve the integrity of the unique DNA present in these spc molecules.

Another point to be made is that even though the host genome contained upwards of $10^5$ endogenous repetitive sequences homologous to the ones present in Rml, there is no indication that integration occurred in these sequences. Obviously the small number of events that we looked at does not exclude that such events would occur at a lower frequency than illegitimate integration. It is possible that the length and degree of homology between the repetitive sequences of Rml and that of the host DNA were not sufficient to favor homologous integration. It has been shown however that homologous recombination between endogenous chromosomal repetitive sequences can occur (41, 42, 45, 46). It is also possible that homology searching is not the rate limiting step that will determine if integration is to occur by homologous or illegitimate recombination. This would explain why the frequency of homologous integration in mammalian cells is very low compared to bacteria and yeast, even when taking into account genome size (47, 48, 49, 50).

It has been postulated that DNA transfected in the presence of carrier forms a high molecular weight structure designated transgenome or pekelosome and that it is these structures that integrate into the host genome (14, 15, 16). Transgenomes are the product of ligation between carrier and the transfected DNA and result in molecules of several hundred Kbp in size. If integration of transfected DNA was necessarily mediated by transgenomes then this would preclude drawing conclusions on the interaction of specific exogenous DNA with the host genome. In this paper we have demonstrated that Rml integrated on its own, without the formation of transgenomes. Thus transgenomes are not a necessary intermediate for the integration of transfected DNA.

A number of authors have reported that integration of exogenous DNA resulted in genome rearrangements (15, 37, 51, 52, 53, 54, 55). However the nature and the extent of these rearrangements were undetermined nor had it been established if host sequences were lost as a result of integration. In all cases, what had been determined as the minimum length of the rearranged
host sequences was equal or smaller to what had been integrated, except in one instance where the rearrangement was of at least 17 Kbp for an insertion of about 8 Kbp (37). This represented the longest rearrangement so far determined. Thus from these results it could be hypothesized that integration involved a simple replacement of host sequences by an incoming linear molecule causing a deletion of similar length as the insertion. Our analysis of the integration site of clone 11.6 establishes that indeed integration can result in the deletion of host sequences. However the extent of the rearranged host sequences, which can be more than 90 Kbp for an insert of 6 Kbp is incompatible with a linear insertion-replacement model as suggested above. Such extensive rearrangements could be explained by an integration mechanism in which the incoming exogenous DNA replaces a looped structure of host cellular DNA.

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LITERATURE CITED
