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REVERSAL OF A NEUROSPORA TRANSLOCATION BY CROSSING OVER INVOLVING DISPLACED rDNA, AND METHYLATION OF THE rDNA SEGMENTS THAT RESULT FROM RECOMBINATION

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ABSTRACT

In translocation *OY321* of *Neurospora crassa*, the nucleolus organizer is divided into two segments, a proximal portion located interstitially in one interchange chromosome, and a distal portion now located terminally on another chromosome, linkage group I. In crosses of Translocation × Translocation, exceptional progeny are recovered nonselectively in which the chromosome sequence has apparently reverted to Normal. Genetic, cytological, and molecular evidence indicates that reversion is the result of meiotic crossing over between homologous displaced rDNA repeats. Marker linkages are wild type in these exceptional progeny. They differ from wild type, however, in retaining an interstitial block of rRNA genes which can be demonstrated cytologically by the presence of a second, small interstitial nucleolus and genetically by linkage of an rDNA restriction site polymorphism to the mating-type locus in linkage group I. The interstitial rDNA is more highly methylated than the terminal rDNA. The mechanism by which methylation enzymes distinguish between interstitial rDNA and terminal rDNA is unknown. Some hypotheses are considered.

THE single nucleolus organizer region (NOR) of *Neurospora crassa* is located at the left end of linkage group V, which corresponds to the short arm of chromosome *2* (BARRY and PERKINS 1969). The NOR is terminal except that a small cytologically recognizable satellite is present in some strains (MCCLINTOCK 1945). The NOR consists of an array of about 200 tandem, directly repeated copies of the gene specifying 17 S, 5.8 S and 25 S ribosomal RNAs, with the individual units separated by nontranscribed spacer regions (FREE, RICE and METZENBERG 1979; COX and PEDEN 1979; RUSSELL *et al.* 1984).

In translocation *T(I → V)OY321*, the NOR has become separated into two

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parts (PERKINS, RAJU and BARRY 1984). This resembles the translocation used by MCCLINTOCK in her 1934 study of the nucleolus organizer of maize. A distal block of rDNA repeats, with the satellite, is interchanged with a long terminal segment of linkage group IL (see Figure 1). Both the interstitial and terminal NORs of *OY321* are capable of forming nucleoli. In crosses homozygous for the translocation, genetic markers that flank the breakpoints show the expected rearranged linkage relations: mating type and other markers located proximal to the IL breakpoint segregate independently of genes in linkage group V and of genes in the translocated IL segment.

We report here the occurrence of exceptional progeny having apparently Normal chromosome sequence. These progeny originate from crosses in which both parents were *OY321* translocation sequence. Cytological and molecular evidence indicates that reversion of the translocation has occurred by crossing over between homologous repeats in the translocated terminal NOR segment and the interstitial proximal NOR segment, and that the apparently normal product differs from wild type in retaining an interstitial block of rRNA genes linked to mating type in linkage group I. For this reason, the term "Quasinormal Sequence" (QNS) will be used to refer to "revertant" strains of this constitution. In QNS strains, the interstitial NOR segment in I is capable of organizing a small second nucleolus in addition to the restored nucleolus with satellite at the end of linkage group V. The rDNA in the interstitial position is resistant to cleavage by restriction endonucleases which fail to cut if particular cytosines in their recognition sites are methylated. rDNA from QNS strains grown in the presence of a methylation inhibitor (5-azacytidine) was not resistant to restriction, confirming that the displaced rDNA is hypermethylated.

MATERIALS AND METHODS

Strains and genetic methodology: The origin and characteristics of translocation *T(I → V)OY321* have been described by PERKINS, RAJU and BARRY (1984). The established structure is shown in Figure 1. For methodology and theory underlying the identification and scoring of chromosome rearrangements such as *OY321*, see PERKINS and BARRY (1977). Gene markers are listed in the legend of Figure 1. For information on individual markers, see PERKINS *et al.* (1982). Oak Ridge wild types OR23-1VA and ORSa and the aconidiate Oak Ridge-derived *fluffy* testers fl^{P}A and fl^{P}a were used as Normal sequence standards. Caffeine resistance is scored reliably at 25°, not 34°, on agar medium containing 2 mg of caffeine per milliliter. Construction of certain duplication strains involving the nucleolus organizer is described in the legends of Figures 8 and 9.

In searching for recombinants having changed sequence, progeny were obtained from structurally homozygous crosses—either *OY321* × *OY321* or QNS × QNS. Each progeny strain was tested for sequence by crossing it to a Normal sequence *fluffy* tester and scoring the resultant ascospores for presence or absence of a class of defective ascospores diagnostic of the *OY321* rearrangement sequence. Tests were carried out on slants of synthetic crossing medium in small (10 × 75 mm) tubes and were scored by examining ascospores ejected to the wall of the tube 10 days after fertilization, as described previously (PERKINS and BARRY 1977).

Cytology: Crosses were incubated at 25°. The number of nucleoli in individual nuclei was examined using hematoxylin, which stains nucleoli intensely [see RAJU (1978) and RAJU and NEWMEYER (1977) for method]. For determining number of nucleoli per

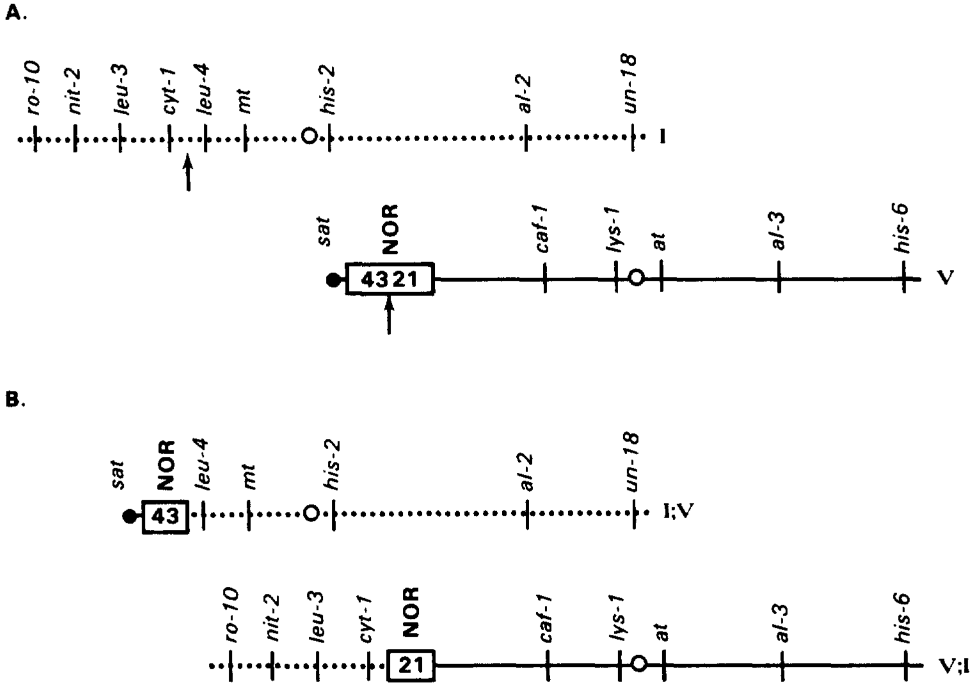


FIGURE 1.—Origin and structure of translocation *OY321*. A, Normal sequence. B, Translocation sequence. Interchange points are indicated by vertical arrows, centromeres by open circles. Symbols: *al*, albino; *at*, attenuated morphology; *caf*, caffeine resistant; *cyt*, cytochrome deficient; *his*, histidine requirement; *leu*, leucine; *lys*, lysine; *mt*, mating-type alleles *A* or *a*; *nit*, nitrate utilization; *NOR*, nucleolus organizer region; *ro*, ropy morphology; *sat*, nucleolus satellite; *un*, unknown heat-sensitive function. Markers are shown for orientation, and interval lengths may be disproportionate. Linkage group arms IR and VR are shortened relative to other regions. rDNA segments in the NOR are numbered 1–4, proximal to distal.

nucleus, 4-day-old crosses were sometimes moved to 5° for 2 or 3 days before fixing the perithecia, with the object of increasing the number of pachytene nuclei in which two separate nucleoli could be seen. Presence of the nucleolus satellite was determined using aceto-orcein (BARRY 1966), which stains the nucleolus lightly, if at all; strains to be scored were crossed to testers known to be *sat*$^+$, and several asci in each testcross were examined at pachytene for the presence of either one or two satellites (BARRY and PERKINS 1969).

DNA isolation and restriction analysis of genomic DNA: Isolation of DNA, restriction digestions, gel electrophoresis and Southern hybridizations were done as previously described (METZENBERG *et al.* 1985). The detection of various regions of rDNA employed nick-translated plasmids pKD002, pKD015, pKD018 and pRW615b, the last having been kindly provided by PETER J. RUSSELL. The map of the rDNA of Oak Ridge wild type and of *sat*$^-$ is shown in Figure 2, with the extent of insertions in each of these plasmids indicated below the map.

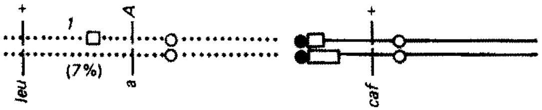
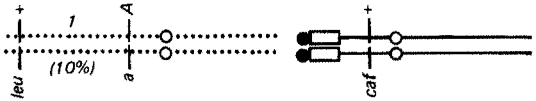
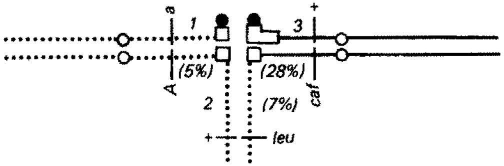
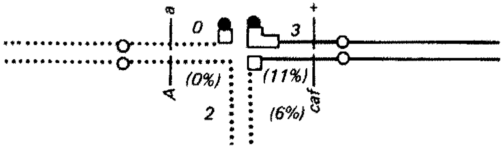
5-Azacytidine treatment: Conidia (~5 × 10^6/ml) were germinated at 30° with vigorous shaking for 18 hr in Vogel's medium N supplemented with 2% sucrose and 24 μM freshly prepared 5-azacytidine.

RESULTS

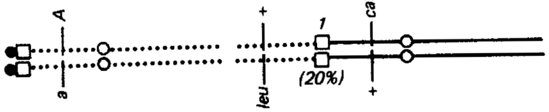
As expected, progeny from crosses of Translocation *OY321* × Translocation *OY321* normally retain the translocation sequence of the parents. Data from

TABLE 1

Linkage of markers in crosses involving the exceptional retranslocated QNS-1 strain, compared with crosses involving the *OY321* translocation from which it originated, and with a Normal sequence control

		Euploid progeny						
		Recombinations						
Zygote genotype, numbering of regions, and recombination (%)	Parental combinations	Singles region 1	Singles region 2	Singles region 3	Doubles regions 1 and 2	Doubles regions 2 and 3	Doubles regions 1 and 3	Triples regions 1,2,3
Cross 1: *QNS-1 A* × Normal *leu-3 caf-1*^{R}*a*								
	38	1						
	20	4						
		(*caf-1* is independent of *leu-3*: 36/72 recombination.)[a]						
Cross 2: Wild-type OR *A* × Normal *leu-3 caf-1*^{R}*a*								
	37	5						
	34	3						
		(*caf-1* is independent of *leu-3*: 42/72 recombination.)[a]						
Cross 3: *QNS-1 A* × *T(I→V)OY321 leu-3 caf-1*^{R} *a*								
	25	0	0	4	0	1	1	1
	15	0	1	8	0[b]	0	0	1
	(Plus 18 inferred Duplications.)[b] (All markers are linked.)							
Cross 4: Normal *A* × *T(I→V)OY321 leu-3 caf-1*^{R} *a*								
	20	0	2	3	0	0	0	0
	25	0	0	2	0	1	0	0
	(Plus 15 inferred Duplications.)[c] (All markers are linked.)							

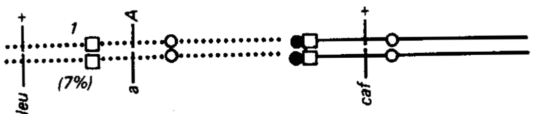
Cross 5: *T(I→V)OY321 caf-1 A* × *T(I→V)OY321 leu-3 a*



81	19
72	20

(*caf-1* is linked to *leu-3:* 39/192 recombination.)
(Mating type is independent of *leu-3*; 87/192.)

Cross 6: *QNS-1 A* × *QNS-7 leu-3 caf-1^R a*



198	14
224	19

(*caf-1* is independent of *leu-3*: 52/99 recombination.[a])
(Mating type is linked to *leu-3*: 33/455.)

Numbers in the body of the table represent euploid progeny, which are fertile in testcrosses. Where duplication progeny were recovered (crosses 3 and 4), the numbers are given in parentheses. Recombination percentages are calculated from the euploid progeny only. The top number of each pair of complementary classes represents progeny having the allele of the leftmost linked locus that is uppermost in the zygote genotype diagram. Segments normally in linkage group V are shown as solid lines, and segments normally in linkage group I as dotted.

Black ascospores were isolated at random to individual slants. All progeny were scored for chromosome sequence in crosses 3, 4 and 6. This was accomplished by crossing each progeny to Normal-sequence fluffy testers and examining the ascospores produced. If 95% or more of ejected ascospores are black, the tested strain is judged Normal sequence, whereas 75% black signifies Translocation sequence. This scoring was used to determine the location of crossovers in crosses 3 and 4. In crosses of Normal sequence × *T(I→V)OY321*, viable duplication (Dp) progeny are produced when the two lower centromeres segregate together (PERKINS, RAJU and BARRY 1984). The duplication progeny are initially semibarren; perithecia are produced in the test crosses, but fewer than normal numbers of ascospores are produced or ejected from young perithecia. Cross 4 is an example.

[a] Recombination of *caf-1* and *leu-3* in crosses 1, 2 and 6 was determined using progeny isolated at a different time from those scored for *leu-3* and mating type.

[b] Scoring of *Dp* vs. *T* was not possible by barrenness in this cross. Most of the inferred Duplications score as a fertile A T Leu$^+$ Caf^R. These are interpreted to have originated by segregation of the two lowermost chromosomes together, to give *A leu$^+$/leu-3 caf-1^R* Duplications scoring as Leu$^+$. Fertility may have resulted from deletion of the IL segment bearing *leu$^+$*, so as to restore translocation sequence. Restoration of fertility by deletion of duplications has not been investigated directly for Duplication progeny from QNS × *T(I→V)OY321*. The suggested sequence of events is based on what is known of unstable duplications from other rearrangements such as *T(I→V)ALS182* and *T(I→V)AR190* (PERKINS and BARRY 1977). In cross 3, double crossovers in the short intervals 1 and 2 would be required to produce nonduplication euploid progeny that were *A T leu-3$^+$caf-1^R*, so as to simulate the putative Duplications. This is highly unlikely because progeny of phenotype A T Leu$^+$Caf^R, which simulate 1,2 double crossovers, constituted a major class (14 of the 18 inferred Duplications among a total of 75 progeny) in a cross where there was no single crossover in region 1 and only one single crossover in region 2. Also, the complementary euploid 1,2 double crossover class *a N leu-3$^+$caf-1^S* was absent. (*N* signifies Normal sequence.) The only tenable explanation is that the A T Leu$^+$Caf^R progeny originated as Duplications, and the missing complementary class is deficient and inviable. This was confirmed by crossing six of the A T Leu$^+$ Caf^R putative Duplications by wild type. Leu$^-$ progeny were recovered from five of the six crosses. Barren Duplications in cross 4 were also predominantly A Leu$^+$Caf^R.

[c] For 13 of the 15 inferred Duplications, scoring of *Dp* vs. *T* was based on barren perithecia in test crosses. The other two were inferred to be Duplications from marker phenotypes, as in cross 3.

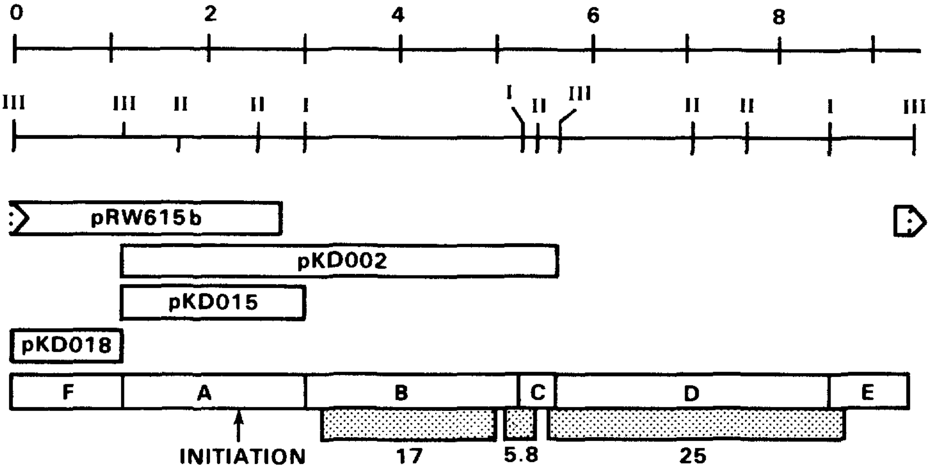


FIGURE 2.—Skeleton restriction map of the ribosomal DNA of Oak Ridge wild type and of *sat*⁻, showing only those sites pertinent to this work. At the top is a distance scale in kilobase pairs. The enzyme symbols used are I, *Eco*RI, II, *Hinc*II; III, *Hind*III. Those vertical marks on the restriction map extending above the line are sites in Oak Ridge wild type, those below the line are sites in *sat*⁻. The extent of each of the four plasmids used as probes in this work is shown below the map. pRW615b is bounded on its left (to the far right on this map) by a *Sal*I site and on its right by a *Pst*I site. The other three plasmids are bounded by sites shown in the map above them. Fragments F, A, B, C, D and E are as in RUSSELL *et al.* (1984). The regions corresponding to mature rRNA species (shaded bars; FREE, RICE and METZENBERG 1979) and to initiation of transcription (TYLER and GILES 1985) are indicated at the bottom.

representative *T* × *T* crosses were presented in table 1 of PERKINS, RAJU and BARRY (1984). From one of the homozygous translocation crosses, a single exception was found unexpectedly among 97 progeny. The exceptional strain no longer behaved as a translocation, but had apparently reverted to the Normal wild-type sequence ("Quasinormal Sequence," QNS). When QNS was crossed with a Normal sequence tester, wild-type linkages were restored (Table 1; compare cross 1 with cross 2 Normal control). The cross of QNS × Normal sequence produced no more than the normal background frequency of aborted ascospores, indicating that the parents were isosequential.

When QNS was crossed with an *OY321* translocation strain as tester (Table 1, cross 3), it gave linkage data like those obtained from crosses of Normal × Translocation (cross 4), and it produced 20–25% aborted white ascospores, which is characteristic of crosses of Normal sequence × Translocation sequence. This behavior is quite unlike what is expected of a cross homozygous for the translocation (cross 5). Chromosome sequence of QNS was thus indistinguishable from wild type by each of two criteria: (1) expected genetic linkages and (2) the production of aneuploid meiotic products when QNS was crossed with the original translocation.

The above data are for the first exceptional strain found, QNS-1. Other independent occurrences of retranslocation were subsequently found and designated QNS-2 through QNS-7 (Table 2). The overall frequency of retranslocation was 0.4% among random ascospores from crosses homozygous for the

TABLE 2

Origin of progeny having Quasinormal Sequence (QNS) from crosses homozygous for translocation *OY321* (*T*)

Parent genotypes	No. of progeny		QNS designation[c]
	Translocation[a]	QNS[b]	
T A × *T nit-2 leu-3 a*	96	1	QNS-1
	74	0	
	86	0	
	87	0	
	194	2	QNS-2, QNS-3
T A × *T a*	90	0	
	153	0	
	130	0	
T A × *T leu-3 caf-1 a*	190	2	QNS-4, QNS-5
	175	0	
T A × *T nit-2 leu-3 caf-1 at a*	286	2	QNS-6, QNS-7
Total	1561	7 (0.4%)	

Numbers on successive lines represent progeny isolated at different times either from the same cross tube or from crosses set at different times using the same parents.

[a] Scored on the basis of 75–80% black ascospores, 25–20% defective white ascospores, in test crosses × Normal-sequence testers; 90–95% black ascospores, 10–5% defective white ascospores, in testcrosses × translocation *OY321*.

[b] Scored on the basis of 90–95% black ascospores, 10–5% defective white ascospores, in test crosses × Normal-sequence testers; 75–80% black ascospores, 25–20% defective white ascospores, in testcrosses × translocation *OY321*.

[c] QNS-1 (FGSC no. 5380), -2, -3, -4 and -5 were *nit*$^+$ *leu*$^+$ *caf*^{S} *A*. QNS-6 (FGSC no. 5381) and -7 were *nit-2 leu-3 caf-1 at a*. QNS-1 was detected by D. D. PERKINS, QNS-2 and -3 by A. M. RICHMAN, QNS-4 and -5 by J. L. CAMPBELL, QNS-6 and -7 by V. C. POLLARD. (FGSC signifies Fungal Genetics Stock Center.)

translocation. Each of the retranslocations resembled QNS-1 in its crossing behavior and in having Normal sequence linkages restored. An intercross of QNS-1 × QNS-7 (Table 1, cross 6) behaved like Normal × Normal.

In addition to scoring the overall percentages of defective ascospores, crosses of QNS strains were also scored for the frequencies of individual asci containing given numbers of defective ascospores (Table 3). The QNS strains behaved like Normal sequence in all these crosses.

Hypothesis: Restoration of an apparently Normal sequence might be achieved by legitimate crossing over if the displaced block of rDNA genes sometimes paired with homologous sequences of the interstitial block (Figure 3). The Normal wild-type gene sequence would be restored in the recombinant chromatids that resulted from a crossover. Chromosome sequence of the exceptional crossover progeny would differ from wild type in only one respect—retention of an interstitial block of rDNA cistrons at the site of the original *OY321* breakpoint in IL, which would have originated from the repeat units from an internal region of the NOR (here designated regions 2 and 3). This interstitial block might be large or small, depending on the register between the multiple copies in which pairing occurred. The restored terminal NOR of

TABLE 3

Percentages of asci containing various numbers of inviable (white) deficiency ascospores in crosses of the exceptional retranslocated QNS strains, compared to the *OY321* translocation strains from which they originated

	Black:white ascospores in individual asci					No. of asci scored
	8:0	6:2	4:4	2:6	0:8	
T(I→V)OY321 × *T(I→V)OY321*	94	5	1	0	0	128
T(I→V)OY321 × *Normal sequence*	42	43	14	1	0	130
T(I→V)OY321 × *QNS-1*	27	48	20	4	2	120
Normal sequence × *Normal sequence*	90	9	1	1	0	152
QNS-1 × *Normal sequence*	89	7	2	2	1	122
QNS-2 × *Normal sequence*	95	4	1	0	0	146
QNS-3 × *Normal sequence*	88	7	4	0	1	101
QNS-4 × *Normal sequence*	96	3	1	0	0	178
QNS-5 × *Normal sequence*	84	11	5	1	0	128
QNS-6 × *Normal sequence*	94	2	4	0	0	126
QNS-7 × *Normal sequence*	89	7	4	1	0	169

VL should be complementary in size to the interstitial NOR of IL, reflecting pairing register at the time of crossing over.

Several predictions from this model can be tested:

1. The exceptional progeny with restored quasinormal sequence should be capable of producing a second nucleolus that is interstitial, in addition to a terminal nucleolus. QNS strains of independent origin might be expected to differ from one another in size-ratios of nucleoli in the two locations. Relative size of the two nucleoli is not predictable *a priori* because differences in register at time of crossing over might result in different numbers of genes in the two positions from one occurrence to another, and because transcription might be differentially regulated.
2. Because translocation *OY321* originated in Oak Ridge (OR) wild-type background, and subsequent stockbuilding crosses employed strains of the same genetic background, the recombined terminal nucleolus should bear a satellite.
3. Some of the ribosomal DNA should be linked to the mating-type locus in crosses between QNS and a Normal sequence strain. This linkage should be detectable if the Normal sequence parent contains a restriction-site polymorphism distinguishing it from the rDNA of the Oak Ridge wild type from which QNS and its *OY321* parents were derived.
4. Because QNS strains contain homologous rDNA repeats in two chromosomes, they should be capable of undergoing interchromosomal recombination comparable to that which produced them in the first place, so as to retranslocate distal IL onto the NOR in VL and restore the original *OY321* translocation sequence.

Observations so far are consistent with the first three predictions. Tests of the fourth have been negative. No restoration of translocation sequence has yet been found, although 1012 progeny have been tested from crosses of QNS

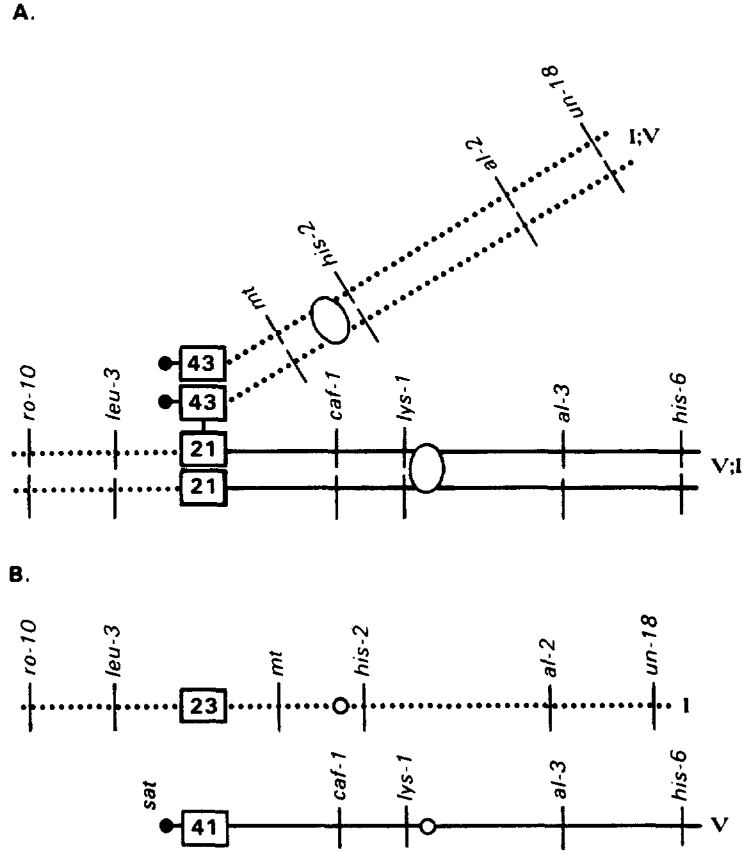


FIGURE 3.—Inferred origin of the exceptional retranslocated QNS progeny by meiotic crossing over between homologous rDNA segments in the interstitial and terminal positions, in a cross homozygous for the translocation. A, Crossing over between homologously paired rDNA semgnets of I;V and V;I. B, Resulting Quasinormal Sequence. Ascospores that are QNS would result only if the two complementary crossover chromatids were both delivered to the same meiotic product. rDNA segments are numbered as in Figure 1. About 200 rRNA genes are tandemly repeated in the NOR, and pairing might also occur in registers other than that shown, producing rDNA arrays of unequal size.

× QNS, and 246 progeny have been tested from crosses of QNS × various wild-type strains.

The QNS strain is capable of forming a second, interstitial nucleolus: Asci of QNS × Normal (Oak Ridge) were examined using hematoxylin at pachytene, the stage of the entire life cycle at which nucleoli reach their largest dimensions. In addition to a large terminal nucleolus of approximately normal dimensions, a second, small but distinct nucleolus was present in 1–10% of the pachytene asci. The second nucleolus was seen most frequently (5–10%) when 4-day-old perithecia were kept at 5° for 2 or 3 days before fixing. At 25°, only 1 or 2% of pachytene asci show a small second nucleolus. (These results were based on observation of at least 400 nuclei at each temperature.) The small nucleolus was clearly interstitial in many nuclei (Figure 4A–D), but qual-

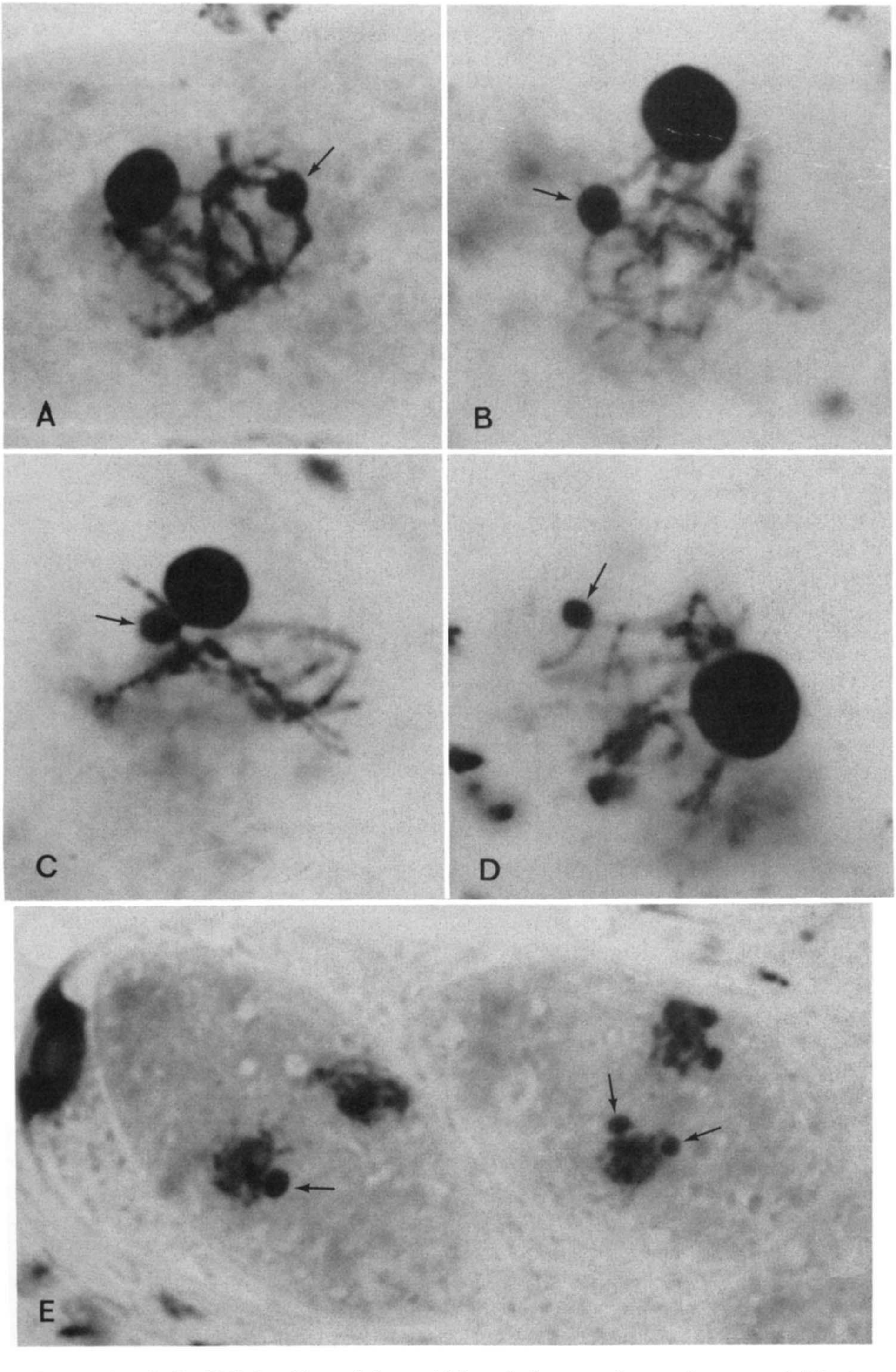


FIGURE 4.—A–D, QNS-1 × Normal. Interstitial nucleolus at pachytene (see arrows). The interstitial nucleolus is much smaller than the main nucleolus at pachytene, but the size difference between the two nucleoli is less obvious at other stages. E, QNS-6 × Normal. Two binucleate sister ascospores. Each of the two interphase nuclei in the right ascospore shows two nucleoli of roughly equal size. The left ascospore shows a single larger nucleolus. (×3000.)

ity of the preparations did not allow the specific chromosome to be identified. In other nuclei, the position of the small nucleolus was obscured by overlying chromosomes, but in no instance did it appear to be terminal. A small interstitial nucleolus could also sometimes be seen in the extended chromosomes at interphase I or II.

A second, small nucleolus might be absent in most pachytene nuclei either because it was not formed or because it had fused with the large terminal nucleolus. In the latter case, an association of chromosome *1* (linkage group I) with the large nucleolus would be expected; long and short segments of chromosome *1* should appear attached to the nucleolar surface, in addition to the terminal attachment of chromosome *2* (linkage group V). Information on this point is not clear. In some of the pachytene nuclei where a separate small interstitial nucleolus was not visible, a second chromosome was indeed associated interstitially into the main nucleolus. The identity of the second chromosome was not established, however. The nucleolus becomes opaque with our hematoxylin staining procedure, so as to obscure underlying chromosomes. Observations with orcein staining, where the nucleolus stains faintly, suggest that a second nucleolus is not visible with this stain on QNS chromosome *1* at pachytene, even when the chromosome *1* bivalent lies free of the major nucleolus.

Fusion of nucleoli at pachytene is the rule rather than the exception in crosses homozygous or heterozygous for translocation *OY321*, where two active NORs are known to be present. Nucleoli fuse less frequently in nuclei undergoing postmeiotic mitosis than in meiotic prophase nuclei in these crosses. This is true especially after the fourth nuclear division in the ascus, which occurs in the young ascospores; 28% of 2-NOR nuclei display two nucleoli in ascospores from *OY321* × *OY321*, and 15% (93 of 624) do so in *OY321* × Normal (PERKINS, RAJU and BARRY 1984; N. B. RAJU, unpublished observations). Nuclei were therefore examined in young binucleate ascospores from QNS × Normal wild type (Figure 4E). Of 512 nuclei examined (32 asci), 256 nuclei were expected to carry two nucleolus organizers, but only eight nuclei showed two nucleoli—a mere 3% of two-NOR nuclei.

It is not clear why cold treatment of developing asci before fixation increases the proportion of pachytene nuclei that show a small interstitial second nucleolus. Perhaps the incipient nucleoli do not readily fuse at this temperature, or perhaps low temperature may somehow promote the formation of the second nucleolus.

A second nucleolus was not observed among several hundred nuclei in asci of control crosses, using wild-type parents and following the same protocols (including the 5° treatment), with the following exception: Nondisjunction of the nucleolus organizer chromosome may give rise to two nucleoli in one or two meiotic products in rare control asci, but the complementary products then contain no nucleoli at all, unlike the situation with *OY321* or QNS.

We conclude that QNS-1 differs from wild type in being able to form a second, small interstitial nucleolus at pachytene or in nuclei of young ascospores. The frequency with which this can be observed is low. Our observations are inadequate to establish whether the reason for absence of a second nucleolus in most of the two-NOR nuclei is that it fails to form, or that fusion has occurred between minor and major nucleoli. In at least some pachytene nuclei, a second chromosome is seen to be associated interstitially with a single large nucleolus.

QNS resembles the wild-type strain from which translocation *OY321* originated in having a nucleolus satellite, and with respect to restriction sites in the nontranscribed rDNA spacers: The nucleolus satellite is present in Oak Ridge wild-type strains (*sat*$^+$) (ST. LAWRENCE 1953), but is absent in strains collected from nature and in certain other laboratory strains (*sat*$^-$). To check for the presence of a satellite in QNS, pachytene chromosomes were examined cytologically in a cross of QNS × wild-type strain ORSa, which contains a satellite. Two satellites were observed on the surface of the nucleolus with sufficient consistency to conclude that QNS has a satellite.

Within the sensitivity of detection, all the nontranscribed rDNA repeat spacer regions within any single Neurospora strain have restriction sites in identical locations, presumably reflecting identity of DNA sequence at other sites as well (FREE, RICE and METZENBERG 1979). Strains originating from different sources in nature show characteristic differences in the nontranscribed spacers, however (RUSSELL *et al.* 1984). In laboratory strains, the presence or absence of the satellite is completely correlated with the presence (type II) or absence (type I) of a particular *Hin*dIII site in the nontranscribed spacer (see Table 4). In addition, the repeat unit of the *sat*$^-$ strain has a *Hin*cII site where none is present in the *sat*$^+$, Oak Ridge-derived strains. One or the other or both of these sites were used diagnostically to see whether the restriction pattern was grossly changed by the process of translocation itself and to follow the segregation of rDNA in crosses. As expected, the restriction pattern of *Hin*dIII + *Eco*RI-cut rDNA from QNS, revealed by probing with pKD002 or pKD018, was not grossly different than the pattern from other *sat*$^+$, Oak Ridge-derived strains. However, some more subtle differences were revealed by an outcross to a *sat*$^-$ strain.

Some rDNA in QNS is linked to the mating-type locus in linkage group I: The diagram of the cross shown in Figure 5 predicts that about one-half of the progeny strains which carry the *A* mating-type allele will be *sat*$^-$ and will carry the rDNA normally associated with it (type I). However, among these *A* type-I progeny, all except the minority that have crossed over between mating type and the breakpoint will also carry at least a small amount of the rDNA normally associated with *sat*$^+$ (type II). For mnemonic value, we shall refer to these as carrying "type I(1,2,3,4) type II(3,2)" rDNA, where the arabic numerals increase as they proceed away from the centromere in the normal sequence chromosome. Of the *a* mating-type progeny that carry type I rDNA, few should also carry any type II rDNA. To test this prediction, we examined 30 progeny from a cross of QNS × *sat*$^-$. These were scored for mating type, and filters from Southern transfers bearing *Hin*dIII + *Hin*cII digests of total genomic DNA from these progeny were probed with pKD018 and were classified as having rDNA of predominantly either type I or type II. Those which

TABLE 4

Correlation of restriction pattern with presence of the nucleolus satellite in wild-type *N. crassa* strains

Strain	Fungal Genetics Stock Center no.	Presence of satellite	Type of *Hin*dIII restriction pattern[a]
Lindegren A	853	−	I[b]
Beadle and Tatum 25a	353	−	I
Beadle and Tatum 1A	354	+	II
Abbott 4A	1228	+	II
Abbott 12a	351	+	II[b]
Emerson E5256A	424	+	II
Emerson E5297a	627	−	I
Oak Ridge 74-OR23-1VA	2489	+	II
Oak Ridge ORSa	2490	+	II
Oak Ridge 74-OR8-1a	988	+	II
sat$^-$ a	945	−	I
Costa Rica A	851	−	I
Puerto Rico 18a	429	−	I
Mauriceville-1c A	2225	−	I

[a] Type I contains a single *Hin*dIII site in the nontranscribed spacer region. Type II contains two *Hin*dIII sites.

[b] Lindergren A was reported by RUSSELL *et al.* (1984) to contain two *Hin*dIII sites in the nontranscribed spacer region, and Abbott 12a was reported to contain one site, in contradiction to the results reported here. The present results are correct, and the contrary results reported in figure 1 of RUSSELL *el al.* were due to typographical transcription errors.

had predominantly type I were examined closely for the presence of a minority component of type II rDNA. The example shown in Figure 6 (lanes 3 and 6) [see also Figure 7 (lane 6)] and the results summarized in Table 5 confirm the presence of QNS of small but variable amounts of type II rDNA linked to mating type. Among the 18 segregants which were predominantly type I and could therefore be scored for the minority rDNA component, 17 were in the parental combination with mating type, and one was equivocal.

Some sequences of interstitially located rDNA in QNS are modified: While digests of QNS segregants with *Hin*dIII + *Hin*cII showed the expected fragments of type II rDNA linked to mating type, the same segregants gave a more complex result when their DNA was digested with *Hin*dIII + *Eco*RI and probed with pKD002. The 11 or 12 that contained type II as well as type I rDNA were expected to show a 1.82-kilobase pair (kbp) *Hin*dIII-*Eco*RI fragment from the OR sequences (fragment A, Figure 2). A fragment of this size could be detected in one of the progeny, isolate no. 17, but not in the remainder. Instead, a small amount of a fragment of about 4.5 kbp that was not seen either in conventional Oak Ridge or *sat*$^-$ parental strains was present in the progeny, including no. 17 (results not shown).

Because the presence of about 200 genomic copies of type I rDNA raises the nonspecific background of genome blots probed with rDNA, we were

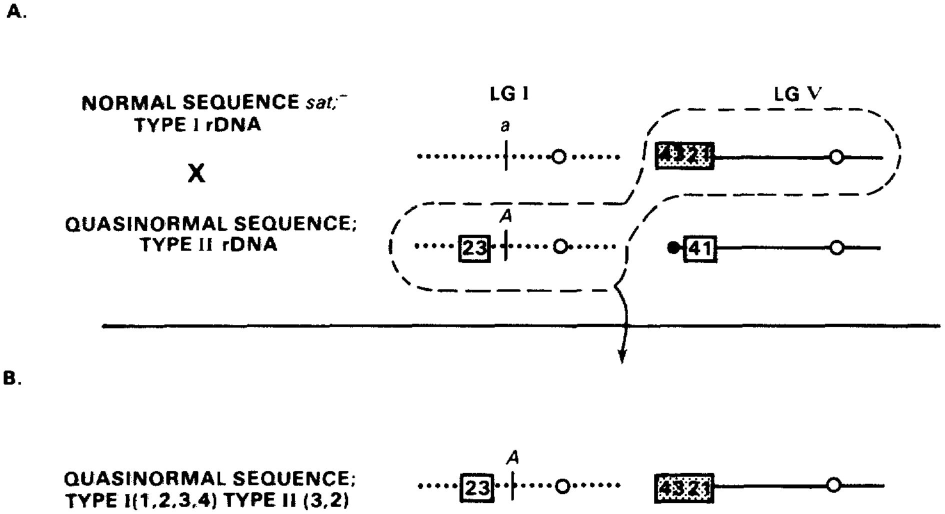


FIGURE 5.—Meiotic assortment of chromosomes in the *sat*$^-$ × QNS-1 cross used to establish linkage of type II spacer sequences to mating type. A, rDNA types in *sat*$^-$ Normal sequence × Quasinormal sequence. B, Diagnostic progeny with both type I and type II rDNA. Nucleolus organizers, satellite and centromeres are indicated as in Figure 1. QNS-1 is *sat*$^+$. Type I rDNA is shaded. For simplicity, only one of each pair of sister chromatids is shown. In the absence of crossing over, independent assortment will give four progeny types, all viable. The diagnostic progeny type is mating-type *A* and contains a majority of type I rDNA and a minority of type II. Crossing over in the short interval between mating type and the interstitial rDNA (3,2) would be required in order to produce mating-type *a* progeny having both type I and type II sequences, or mating-type *A* progeny having only the type I sequence. As shown in Table 5, not more than 1 of 18 informative progeny could have been such a crossover.

uncertain about the degree of confidence with which we could say that the 1.82-kbp fragment was absent. Therefore we did a reconstruction in which genomic DNA from *sat*$^-$ was "spiked" with known amounts of genomic DNA from OR, and the mixture was digested with *Hin*dIII + *Eco*RI and probed as before. We found that the threshold of detection was about one copy of OR rDNA per genome; hence the 1.82-kbp fragment is either absent in most of the isolates or present at no more than one copy per genome. The disappearance of an expected fragment and appearance of an unexpected (and larger) one was consistent with failure to cleave at the *Eco*RI site that normally defines the right end of fragment A and also, perhaps, at the *Eco*RI site defining the boundary between fragment B and the very small fragment C (Figure 2). The 4.5-kbp fragment would then contain the sequences of fragments A + B, or A + B + C. Methylation of cytosine, but not of adenine, has been observed in Neurospora (D. SWINTON and S. HATTMAN, personal communication; BULL and WOOTTON 1984; SELKER and STEVENS 1985), and specifically in Neurospora rDNA (RUSSELL *et al.* 1985). Methylation of the C in the DNA recognition site of *Eco*RI is known to prevent cleavage, while C-methylation of *Hin*cII sites does not do so (MCCLELLAND and NELSON 1985). The effect of C-methylation on cleavage by *Hin*dIII is unknown, but in the present case, the *Hin*dIII sites are cleaved normally. A working hypothesis, then, is that one or

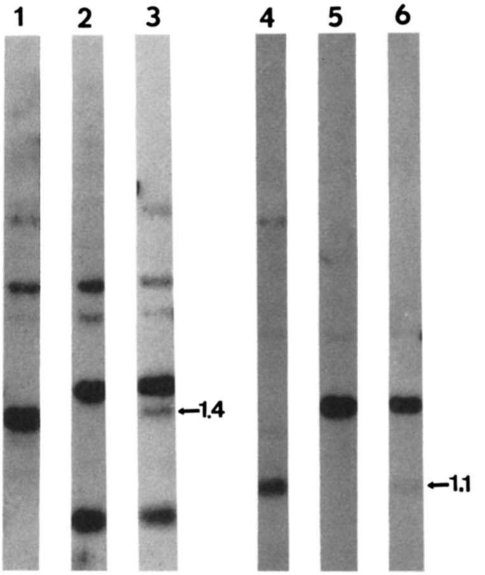


FIGURE 6.—Presence of a minority component of type II rDNA in an isolate from the cross diagrammed in Figure 5. The DNA samples were from the Oak Ridge strain ORSa (lanes 1 and 4) or from *sat*⁻ (lanes 2 and 5) or from isolate no. 17, a putative carrier of type I(1,2,3,4) type II(3,2) rDNA (lanes 3 and 6). The samples were digested with about a tenfold excess of *Hin*dIII + *Hin*cII, fractioned by electrophoresis and probed with nick-translated pKD015 (lanes 1–3) or pKD018 (lanes 4–6). The faint bands seen at about 1.4 kbp in lane 3 and 1.1 kbp in lane 6 are diagnostic of the presence of a minority of type II rDNA.

TABLE 5

Progeny of a cross between Quasinormal Sequence (QNS-1 *A*) carrying rDNA of type II and Normal sequence *sat*⁻ *a* carrying rDNA of type I: linkage of a minority component of type II rDNA to mating type

Mating type	Predominant or sole rDNA type	Minority component of type II present?	Recombination *mt*-rDNA (3,2)	No. of progeny observed
a	Type I	No	Parental	6
a	Type I	Yes	Crossover	0
A	Type I	Yes	Parental	12 (11?)
A	Type I	No	Crossover	0 (1?)
a	Type II			9
A	Type II			3

The expectations of segregants from this cross are described in the text and are diagrammed in Figure 5. The *A*, predominantly type I rDNA segregants showed a wide spectrum in the amount of minority type II rDNA present. The isolate in which it was most abundant, no. 17, is shown in Figure 6. This isolate and no. 21, in which it was present in much smaller but still unequivocal amounts, were taken as prototypes having "high" and "low" amounts of this minority component. In one *A*, predominantly type I rDNA, there seemed to be only a trace of the band characteristic of type II rDNA, and it could not be scored with confidence. None of the *a*, predominantly type I rDNA, samples showed any trace of the type II rDNA.

more *Eco*RI sites that are not methylated under normal circumstances become methylated in the interstitial rDNA of QNS.

Evidence that the modification of interstitial Oak Ridge rDNA (type II) is methylation and that it occurs at several, but not all, cytosines: We investigated the methylation state of rDNA of ORSa, *sat*$^-$ and two QNS isolates, nos. 17 and 21 (see legend of Table 5). To do this, we used other restriction enzymes which fail to cut DNA if certain nucleotides in their recognition sequences are methylated, including *Msp*I, *Hpa*II, *Mbo*I, *Sau*3A and others. The basis for these experiments was as follows: (1) While both *Msp*I and *Hpa*II cleave sequences containing unmodified CCGG, *Msp*I will cleave any CmCGG except GGCmCGG, but *Hpa*II will not do so; conversely, *Hpa*II but not *Msp*I will cleave mCCGG (McClelland and Nelson 1985). (2) While *Mbo*I and *Sau*3A will both cleave GATC sequences, *Mbo*I will also cleave GATmC, but *Sau*3A will not. (3) Growth of cells in 5-azacytidine decreases or eliminates methylation of DNA; unmasking of a restriction site for a methylation-sensitive enzyme, such as *Hpa*II or *Sau*3A, by this agent therefore indicates that the site was previously methylated (Jones 1984; Selker and Stevens 1985).

We compared the cleavage of sites in cloned rDNA with cleavage or noncleavage of the same sites in genomic rDNA, probing for the fragments with nick-translated pRW615b, a plasmid containing most of the nontranscribed region of rDNA (P. J. Russell, personal communication). The genomic DNA was prepared both from strains grown in the usual fashion and from the same strains grown in the presence of 5-azacytidine. We found that, even in wild-type strains, partial methylation of rDNA could be detected, consistent with the findings of Russell *et al.* (1985). In digests of Oak Ridge wild type, primary fragments approximately 230-, 290-, 560- and 970-bp long were apparent in both *Hpa*II and *Msp*I digests. Several minor bands were also observed, of which the most prominent corresponded to a fragment about 1600-bp long. This band was prominent in *Hpa*II digests of DNA from cells grown in ordinary medium, but much less so in *Msp*I digests of the same DNA, or in *Hpa*II digests of DNA from cells grown in the presence of 5-azacytidine. Apparently this fragment results from failure to cleave at a *Hpa*II site that is methylated in a substantial fraction of the cells. Growth of wild type with 5-azacytidine prevents or reduces this methylation, so that the fragment is cleaved by *Hpa*II (data not shown).

Experiments with *Sau*3A and *Mbo*I revealed another restriction site polymorphism between ORSa and *sat*$^-$. In *Sau*3A and *Mbo*I digests of ORSa DNA, fragments of about 1700 and 620 bp were invariably detected (see Figure 7, lanes 1 and 3). The 620-bp fragment was absent in digests of *sat*$^-$ DNA; instead, a fragment of about 1300 bp was detected (not shown). A *Sau*3A fragment of about 2500 bp (roughly the sum of 1700 and 620 bp) was also prominent in *Sau*3A digests of DNA from ORSa grown on ordinary medium, but much less so in *Sau*3A digests of DNA from the same strain grown with 5-azacytidine (Figure 7, compare lanes 1 and 2). The roughly 2500-bp fragment apparently remains uncleaved by *Sau*3A whenever the target sequence

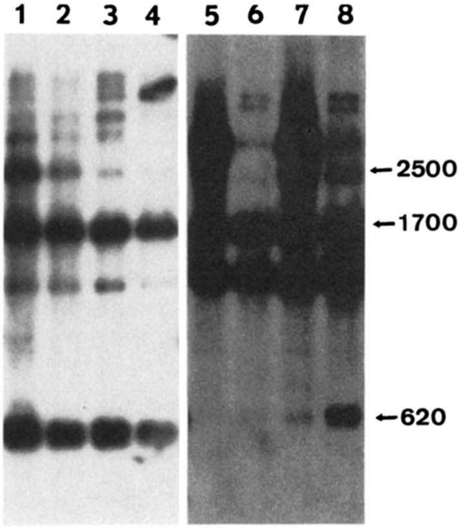


FIGURE 7.—Methylation of rDNA. Neurospora DNA was prepared from young mycelial cultures approaching the end of exponential growth in the presence (lanes 2 and 4) or the absence (lanes 1, 3, 5, 6, 7 and 8) of 24 μM 5-azacytidine as previously described (SELKER and STEVENS 1985). Samples of DNA (approximately 0.5 μg) from the Oak Ridge strain ORSa (lanes 1–4), or from the rDNA type I(1,2,3,4) type II(3,2) isolates no. 21 (lanes 5 and 6) or no. 17 (lanes 7 and 8) were digested with a tenfold excess of *Sau*3A (lanes 1, 2, 5 and 7) or *Mbo*I (lanes 3, 4, 6 and 8). The digests were fractionated by electrophoresis on 1.2% agarose gels, transferred to nylon membranes and probed with nick-translated pRW615b by standard techniques. A band at 620 bp in lane 6 is visible in the original autoradiogram, but was lost in reproduction.

(GATC) contains 5-methylcytosine. Unaccountably, a trace of this 2500-bp fragment even persists in *Mbo*I digests (lanes 3 and 8).

DNA from two of the rDNA type I(1,2,3,4) type II(3,2) isolates from the cross of QNS × *sat*$^-$ were examined in a similar way. In the case of isolate no. 21, which has a "low" type II content (see legend of Table 5), an *Mbo*I digest showed a faint band at 620 bp, whereas a *Sau*3A digest of the same DNA did not show any detectable amount of this band (Figure 7, lanes 5 and 6). When isolate no. 21 was grown in medium containing 24 μM 5-azacytidine and a *Sau*3A digest of its DNA was prepared, the 620-bp fragment could be detected (data not shown). A parallel experiment was done with isolate no. 17. Previous findings had suggested this isolate carries more copies of type II rDNA than the other isolates, which contain both type I and type II rDNA, and that this type II rDNA is less highly methylated. In *Mbo*I digests of DNA from this isolate, a band of 620 bp was seen, and it was more prominent than the one from isolate no. 21 (Figure 7, lane 8). *Sau*3A digests of DNA from isolate no. 17 showed this band even without growth of the strain in 5-azacytidine (Figure 7, lane 7), although the band was more intense in *Mbo*I digests of the same DNA (lane 8).

Similar experiments using the enzyme *Alu*I, which cleaves AGCT sequences but not AGmCT, also revealed a restriction fragment length polymorphism between OR and *sat*$^-$. In this case, however, the type II DNA was detectable in both isolates no. 17 and 21, and growth in 5-azacytidine did not change the amount of the fragment (data not shown). Thus some, but not all, cytosines in the interstitial rDNA are methylated.

We have not investigated the possibility of methylation in parts of interstitial rDNA other than in the regions defined by the plasmids pKD002 (and its subclone pKD015), pKD018 and pRW615b. All of the sites known to be methylated are outside of the region that codes for 17 S, 5.8 S and 25 S rRNA. However, at least the *Eco*RI site that defines the border between fragments A and B (Figure 2) is part of the nascent transcript (TYLER and GILES 1985), and pRW615b extends into the 5′-transcribed region as well. It is possible that the parts of the repeat unit which code for mature rRNAs are also methylated. Such methylation would be much more difficult to detect, however, because the polymorphisms which allowed detection in the present case seem to be largely or completely limited to regions of the repeat unit which do not code for mature rRNA.

Sequences immediately proximal or immediately distal to the nucleolus organizer are not necessary to prevent methylation of rDNA: The finding of methylation of certain sites in the interstitial rDNA of QNS strains raises the question of how the organism "knows" these are mislocated and makes them a target for methylation; or, conversely if rDNA naturally contains target sequences, how are these spared from methylation when the rDNA is in the normal location? Of several possibilities which suggest themselves, one can be seen by reference to Figure 3. This shows that the interstitial rDNA consists of repeat units which are normally internal, here represented as units 2 and 3. A possible hypothesis is that the rDNA is naturally a substrate for methylation, and that the methylation enzyme is processive, tending to begin at one end of a stretch of substrate DNA and proceed toward the other. If this is so, one could then postulate the existence of non-rDNA sequences at the proximal (or distal) end of the nucleolus organizer, which prevent the methylase from getting started. In that case, separation of internal repeat units (2 and 3 in Figure 3) from the terminal units 1 and 4 would remove them from the protection of these postulated methylation-inhibiting sequences, explaining the observed methylation of interstitial rDNA. In principle, this hypothesis could be tested simply by observing whether there is a relatively high degree of methylation of rDNA in the original translocation, *OY321*. In this strain, as depicted, units 1 and 2 remain adjacent to the proximal junction between the nucleolus organizer and the rest of linkage group V, and units 3 and 4 to the distal junction, now associated with linkage group I. No abnormal degree of methylation of rDNA can be seen in this strain. However, this result does not in itself rule out the hypothesis. Units 1 through 4 depict the topology of rDNA, but are quantitatively unspecified. Taken together, they represent about 200 rDNA repeat units, but there are no molecular criteria for judging how many of them are in each of the two locations. If the great majority of them still are adjacent to the sequences which protect them from methylation (say, proximal to unit 1), the small change due to methylation of units 3 and 4 would not be observable. Because the original translocation strain *OY321*

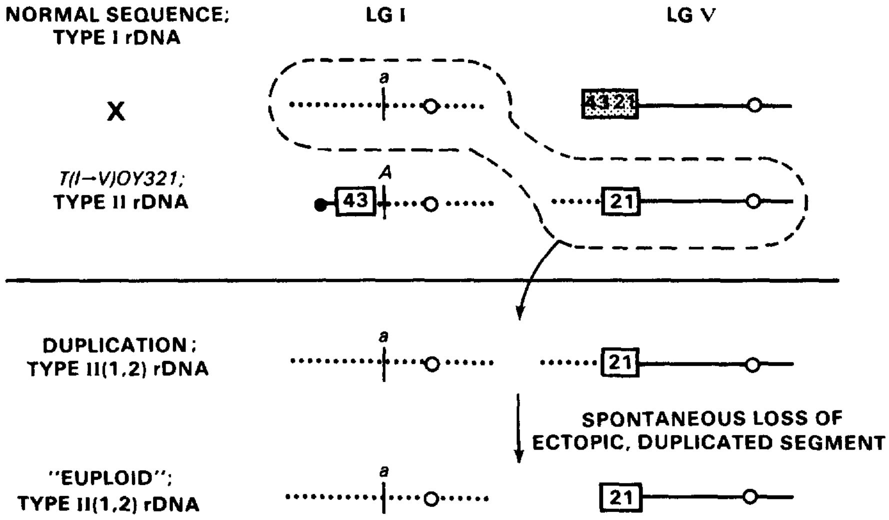


FIGURE 8.—Construction of strains with rDNA type II(1,2). For simplicity, only one of each pair of sister chromatids is shown. The translocation strain *T(I → V)OY321 A*, carrying rDNA of type II, was crossed to a Normal sequence *a* strain carrying rDNA of type I. Nonparental ditype asci with four inviable white spores (putative deficiency progeny) and four viable black spores (putative duplication progeny) were dissected. The putative duplication progeny were verified as recombinants which carried the *a* allele of the mating-type locus and rDNA of type II. These isolates were initially semibarren, as expected of duplication progeny (PERKINS, RAJU and BARRY 1984), but after a few vegetative transfers they became fully fertile. They apparently did so by loss of the duplicated segment of linkage group I distal to the truncated nucleolus organizer (see footnote to Table 1). We tried to detect partial diploids that had retained this segment as a minority vegetative component of several isolates by their ability to "cover" a recessive linkage group I marker (*leu-3*) in a subsequent cross. No such component was detected. Therefore we have depicted the strains from which DNA was isolated and tested as being euploid, except for the absence of units 3 and 4 of the nucleolus organizer.

lacks any rDNA polymorphism, we cannot say if it has more or less methylated rDNA than is found in wild-type strains or in QNS progeny nos. 21 and 17. It is possible, in fact, that all of the strains (ORSa, *sat*$^-$, *OY321*, QNS-1) have comparable levels of methylation, on an absolute basis.

To investigate this further, we prepared two new kinds of translocation-bearing strains. (Diagrams and descriptions of the building of these strains are given in Figures 8 and 9 and their legends). One of these kinds, called "type II(1,2)," allows units 1 + 2 to be examined for methylation in the complete absence of units 3 and 4. A complementary kind of strain having units 3 and 4, but lacking 1 and 2, would be inviable because there are essential genes abutting unit 1. However, the same purpose was realized with strains referred to here as "type I(1,2,3,4) type II(3,4)." Such strains contain a normal nucleolus organizer from *sat*$^-$ and units 3 and 4 from the Oak Ridge *OY321* ancestor. Two questions could then be asked: (1) Do units 3 and 4 contain a substantial portion of the total rDNA? (2) Is the type II rDNA in units 3 and 4 highly methylated?

DNA from ten type I(1,2,3,4) type II(3,4) isolates (pairs of sister spores from

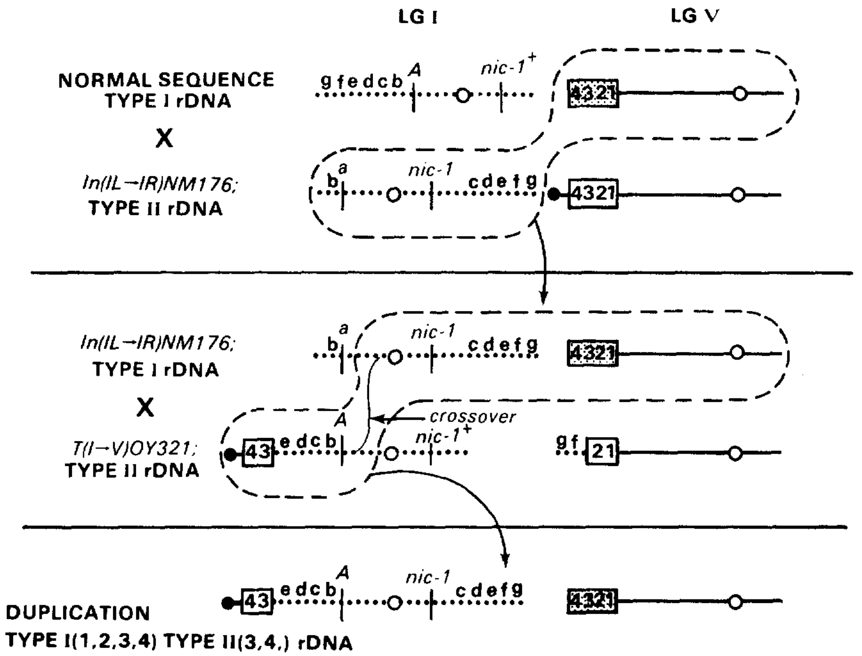


FIGURE 9.—Construction of strains with rDNA type I(1,2,3,4) type II(3,4). For simplicity, only one of each pair of sister chromatids is shown. A prototrophic Normal-sequence strain which had rDNA of type I was crossed to an inversion-bearing strain of the opposite mating type that was *nic-1* and had rDNA of type II. This inversion, *In(IL → IR)NM176*, moves a terminal segment (cdefg) from the left arm of linkage group I to a quasiterminal position on the right arm of the same linkage group. *A* and *a* have their usual meanings as alleles of the mating-type locus, and the lower-case letters b through g symbolize genetic material proceeding away from the centromere in alphabetical order. The proximal inversion-breakpoint is arbitrarily shown between b and c. Unordered tetrads were collected, and those in which all eight spores were black and viable were scored for mating type, *nic-1*, and for Normal sequence *vs.* inversion. An inversion-bearing strain that was *nic-1* and had rDNA of type I was crossed to the translocation-bearing strain *T(I → V)OY321*, which has rDNA of type II. Asci with four white (deficiency) ascospores and four viable black ascospores were candidates for containing duplications which would include units 3 and 4 of the nucleolus organizer. (Such asci can arise by a variety of events, including four-strand double crossovers.) Cultures from the viable black spores were scored for mating type and for the barren trait (evidence of a duplication), for *nic-1* and for rDNA types. Of 12 asci examined in this fashion, five contained sister spores bearing both type I and type II rDNA. Those which carried both types of rDNA were all of mating type *A*. Some of them were *nic-1* like the example diagrammed here, and others were *nic-1*$^+$, reflecting crossover points to the right of the *nic-1* locus.

five different asci) were examined on genome blots after cleavage with *Hin*dIII + *Eco*RI, or cleavage with *Hin*dIII + *Hin*cII, or both. The blots were probed with pKD002 and with pKD015. In every case, the fragment or fragments characteristic of type II rDNA cut with that pair of enzymes was of the same order of intensity as the fragments in the same lane which were characteristic of type I (see Figure 10). At least in these isolates, units 3 + 4 in linkage group I cannot be much less than one-half of the entire nucleolus organizer, and unless amplification has taken place, the same must be true of the original translocation *T(I → V)OY321*. No abnormal amount of methylation was ob-

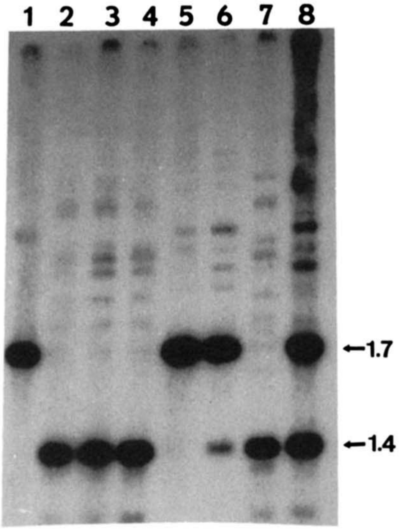


FIGURE 10.—Type I and type II rDNA in strains with various types of translocations. DNA samples from the eight strains were digested with about a tenfold excess of *Hin*dIII + *Eco*RI. The digests were fractionated by electrophoresis and probed with nick-translated pKD015. In this system, the 1.7- and 1.4-kbp fragments are characteristic of type I and type II rDNA, respectively. The DNA samples shown are as follows: lane 1, *sat*⁻; lane 2, ORSa; lane 3, *T(I → V)OY321*; lane 4, QNS-1; lanes 5 and 6, type I(1,2,3,4) type II(3,2) isolates nos. 21 and 17, respectively; lane 7, a type II(1,2) isolate (see Figure 8 and text); and lane 8, a type I(1,2,3,4) type II(3,4) isolate (see Figure 9 and text). In much more heavily exposed autoradiograms, a 1.4-kb band could be seen in lane 5 as well as in lane 6, but at the expense of the clarity of results in other lanes.

servable by *Hin*dIII + *Eco*RI cleavage of the rDNA of the original translocation, nor in that of the type II component (or the type I component) of the type I(1,2,3,4) type II(3,4) isolates. This indicates that merely moving units 3 + 4 into the environment of linkage group I does not cause them to be methylated, nor does taking them away from unit 1 cause them to be methylated.

DNA from three type II(1,2) isolates was similarly examined by cutting with *Hin*dIII + *Eco*RI and probing with pKD002. The patterns were indistinguishable in their methylation from that of Oak Ridge wild type, type II(1,2,3,4) (see Figure 10). Clearly, unit 4 is not necessary in preventing methylation of the rDNA of units 1 and 2.

DISCUSSION

Two central findings are reported here: First, that retranslocation occurs spontaneously by meiotic crossing over involving displaced rDNA sequences in such a way as to restore an essentially Normal sequence (QNS); and second, that displaced rDNA segments at a new chromosomal site are more heavily methylated than those at the normal nucleolus organizer region. Cytological, genetic and molecular evidence has been obtained proving that rDNA is present interstitially in a new second nucleolus organizer region in the retranslocated quasinormal-sequence chromosome *1*, where it is linked to the mating-type locus.

The spontaneous appearance of Quasinormal Sequence (QNS) among meiotic products from crosses homozygous for *OY321* translocation sequence is most simply attributed to meiotic crossing over between homologous rDNA repeat units of the nucleolus organizer segments at the original and translocated positions. It seems unlikely that premeiotic recombination is responsible for the occurrences reported here, because the QNS has been recovered in one or two progeny per cross rather than as clusters or jackpots. Inasmuch as rDNA comprises 7% of the total nuclear DNA in Neurospora (KRUMLAUF and MARZLUF 1980), the occurrence of recombination between displaced rDNA segments is perhaps not surprising.

Rearrangements with one break in the NOR, similar to translocation *OY321*, have been described in maize and in several other organisms (see PERKINS, RAJU and BARRY 1984 for citations). A new example in humans is of both academic and clinical interest (WORTON *et al.* 1984). It is reasonable to anticipate that further rearrangements may be generated by recombination between the displaced rDNA segments in organisms such as these.

Reversion of chromosome rearrangements to wild type: There have been two brief reports of what appears to be spontaneous mitotic reversal of translocations in Saccharomyces (MIKUS and PETES 1982; SUGAWARA and SZOSTAK 1983b). Evidence for retranslocation consisted of high ascospore viability in crosses of the putative revertants with Normal sequence testers, where most asci had all four ascospores viable as expected for isosequential crosses. Further cytological, genetic or molecular evidence has not been presented. In both cases, the original translocations contained rDNA sequences at each breakpoint, similar to translocation *OY321* in the present study.

Other instances of spontaneous reversal of rearrangements have been reported which do not involve displaced rDNA repeats but which may involve other displaced homologous segments. Reinversion has been well documented in Drosophila (GRÜNEBERG 1936, 1937; NOVITSKI 1961; KALISCH 1970). ENGELS and PRESTON (1984) report that those *P*-factor-induced inversions in which both breakpoints retain *P* elements are capable of reverting at high frequency to the original, or approximately original, sequence. These authors suggest that earlier observations of reversion to wild-type sequence in Drosophila may also have involved transposable elements. REUTER, WOLFF and FRIEDE (1985) report reinversions of w^{m4} after *P*-directed mutagenesis in hybrid dysgenic crosses of *D. melanogaster*.

Origin of rearrangements by recombinational joining of displaced homologous segments: The development of techniques for cloning and transformation has made it possible to insert sequences containing a duplicate copy of specific genes into nonhomologous chromosomal loci. Differences in alleles occupying the initial and displaced positions provide a basis for selective screening for recombinants. Technology for accomplishing this has been developed and applied in Saccharomyces (SCHERER and DAVIS 1980; ERNST, STEWART and SHERMAN 1981; POTIER, WINSOR and LACROUTE 1982; MIKUS and PETES

1982; SUGAWARA and SZOSTAK 1983a,b; FASULLA and DAVIS 1984). Reciprocal recombination between the inserted marker and its allele in the normal location in a nonhomologous chromosome is expected to result in reciprocal translocation, whereas gene conversion is not.

Investigations of recombination between genes in displaced segments in yeast have mostly involved selection for mitotic recombinants. However, both chromosome rearrangements and gene conversions have also been recovered meiotically in Saccharomyces (JINKS-ROBERTSON and PETES 1985, 1986) and in Schizosaccharomyces (MUNZ *et al.* 1982; SZANKASI *et al.* 1986).

LEE (1975) has compiled indirect evidence that rejoining of homologous regions plays a role in the origin of rearrangements in Drosophila. The distribution of chromosome-rearrangement breakpoints was compared with the amount of repetitive DNA determined by *in situ* hybridization in euchromatic regions of Drosophila polytene chromosomes. A strong correlation exists.

It is not uncommon for species to have more than one nucleolus organizer region at loci in two or more nonhomologous chromosomes. If one or more NORs are nonterminal, and if crossing over between the displaced homologous rDNA sequences were not repressed in these species, interchanges between the arms that contained NORs would be expected to arise repeatedly. To our knowledge, such interchanges have not been encountered in species such as barley and the castor plant Ricinus which possess two NORs in positions that would allow the detection of translocations. However, a cytological comparison of races of Ricinus revealed many structural variations of the two arms that contain NORs, but few or none elsewhere in the genome (PARIS, SHIFRISS and JELENKOVIC 1980).

In *Escherichia coli,* the seven dispersed ribosomal RNA genes are largely homologous. Recombination between different genes has been shown experimentally to produce duplications and inversions, depending on whether the genes involved have the same or opposite orientations. One already existing *E. coli* lineage differs from other laboratory strains in having an inversion that apparently arose by recombination between two oppositely oriented rDNA genes (HILL and HARNISH 1981).

Crossing over within the rDNA when NORs are in Normal sequence: If crossing over can occur in translocation strains between rDNA segments that are in different chromosomes, it might be expected to occur even more frequently between homologous rDNA segments in NORs that are not displaced. This has been investigated in Drosophila, and frequencies up to 1% have been reported in female meiosis (BONCINELLI *et al.* 1983; SHALET 1969; HILLIKER, APPELS and SHALET 1980). Crossing over also occurs between rDNA of the *X* and *Y* chromosomes in Drosophila bristle selection lines (COEN and DOVER 1983).

Meiotic crossing over in rDNA has also been studied in fungi, using restriction fragment length differences as markers. Crossing over between nonsister chromatids is reduced about two orders of magnitude per unit physical length in ribosomal DNA relative to non-rDNA sequences of yeast (see PETES, SMO-

LIK-UTLAUT and MCMAHON 1982). Meiotic crossing over in rDNA is also reduced relative to non-rDNA in Coprinus (CASSIDY *et al.* 1984).

In *N. crassa,* RFLPs in nontranscribed rDNA spacers are widespread among wild strains (RUSSELL *et al.* 1984). All spacers are alike (within the limits of detection) in any one strain. This would suggest either that recombination does not occur within rDNA in crosses heterozygous for different spacer sequences, or if it does, that resulting heterogeneities are purged by some process that restores homogeneity (see SMITH 1973). COX and PEDEN (1979) have pointed out that the genetic map of the nucleolus organizer arm in Neurospora is markedly shorter than would be expected if crossing over occurred with equal probability in ribosomal and nonribosomal DNA. RFLPs have been used to show that crossing-over frequency is about 1% in the 200-copy array of rRNA genes, where over 60 map units would be predicted from the physical length (R. PETERSEN and P. J. RUSSELL, personal communication). We do not know whether recombination between the displaced rDNA segments in crosses homozygous for translocation *OY321* is similarly repressed.

Basis of the methylation difference: The finding that interstitial rDNA is methylated to an abnormal degree suggested the possibility that certain sequences at one or the other end of the nucleolus organizer normally protect rDNA from methylation, and that removal of rDNA from their protection results in methylation. Experiments designed to test this hypothesis did not support it. An alternative possibility is that methylation enzymes work processively from one or both ends of rDNA. This would be true of even the normal nucleolus organizer, and perhaps of other tandemly repeated DNA as well. However, on this hypothesis, methylation proceeds only through a few repeat units—*i.e.*, a few tens of kilobase pairs—before the next cell division, when it must start again. In a normal nucleolus organizer containing about 2000 kbp of DNA, the methylation of a few percent at one or both ends would not be easily observed in the background of unmethylated rDNA. However, when a few repeat units of rDNA are moved to a separate environment and methylation proceeds from one or both ends, most or all of the interstitial DNA may be methylated. Even this situation (that which presumably exists in the original QNS isolate) would not be easily observable, because it would appear only as a modest increase (perhaps twofold) in a minority band of uncut DNA. It only becomes easily detectable in a strain in which the majority of rDNA of the nucleolus organizer is of one restriction pattern (here, type I), and the interstitial rDNA is of another (here, type II). An additional prediction from this hypothesis is that, if a larger amount of interstitial rDNA is present in some QNS strains, those strains might have some repeat units which are out of reach of the postulated "edge effect." Such may be the case with isolate no. 17 (see above). While we would not want to guess at the number of copies of type II rDNA in the various isolates, this no. 17 clearly has more than the other isolates investigated, and it is also the only one in which the *Hin*dIII-*Eco*RI fragment A and the 620-bp *Sau*3A fragment can be detected. Presumably, the larger number of interstitial rDNA units were generated by some sort of amplification event. If this sort of event can be caused to occur predictably by conditions of culture or temperature, the hypothesis might more easily be put to further test.

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