Use of repetitive sequences to identify DNA polymorphisms linked to *regA*, a developmentally important locus in *Volvox*

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The *regA* locus plays a centrally important role in *Volvox* development by preventing somatic cells from redifferentiating as germ cells; until now, approaches to cloning *regA*, as a preliminary to molecular analysis of its function, have been lacking. Here a novel approach is described that uses repetitive-sequence probes to rapidly identify restriction fragment length polymorphisms (RFLPs) linked to *regA*. Genomic DNA was cut with restriction enzymes having 4-base recognition sequences and then electrophoresed long enough to run most fragments off the gel; the remaining long (1- to 20-kb) fragments were resolved into numerous, reproducibly identifiable bands. On Southern blots of such preparations, six repetitive-sequence probes were used to identify 1232 bands, 24% of which were polymorphic between two closely related strains. Ninety-four RFLPs, for which inheritance patterns have been analyzed, fall into 36 "segregation groups," within which no recombination was observed in the limited progeny sample analyzed. Eight RFLPs cosegregated perfectly with alleles at the mating-type (*mt*) locus. More significantly, four RFLPs exhibited linkage to the *regA* locus, providing a potential starting place for a chromosome walk designed to clone the locus.

[Key Words: *Volvox*; germ-soma development; repetitive sequences; RFLP analysis]

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We are attempting to develop tools for molecular genetic analysis of *Volvox carteri* because the organism's simple pattern of multicellularity [only two cell types, somatic and reproductive, organized in a highly regular pattern within a simple sphere (Starr 1970)] and its accessible genetic system (Huskey et al. 1979a) make it a potentially powerful model for analyzing the genetic regulation of cellular differentiation and establishment of a germ-soma dichotomy (Kochert 1975; Kirk and Harper 1986). In addition, a molecular genetic comparison of *Volvox* with its unicellular and colonial relatives in the order Volvocales (Kochert 1973; Pickett-Heaps 1975), should provide a unique opportunity to gain insights into the molecular basis for the evolution of multicellularity and division of labor. We have selected the *regA* locus as the first *V. carteri* gene for detailed study for two main reasons: First, it is clear that *regA* plays a central role in maintaining the germ-soma dichotomy of *Volvox* because mutation at the locus confers on somatic cells, which otherwise exhibit terminal differentiation, the capacity to redifferentiate as reproductive cells (Starr 1970; Huskey and Griffin 1979; Kirk and Harper 1986). Second, it has recently been postulated, on the basis of very unusual mutational properties exhibited by the gene, that the differential expression of *regA* in somatic and reproductive cells may be regulated by a

sequence rearrangement (Kirk et al. 1987). Cloning of the locus and determination of its sequence in somatic and reproductive cells would not only provide a definitive test of the latter hypothesis, but it also would be an important first step in determining how the gene exerts its profound influence on cellular differentiation. However routes to cloning the locus have been lacking, as its product has not yet been identified, and no conventional markers (which might provide starting points for a "chromosome walk") have yet been found linked to *regA*, despite intensive search (Huskey and Griffin 1979; Huskey et al. 1979a; R.J. Huskey, pers. commun.; K. Huson, J. Harper, and D. Kirk, unpubl.).

The potential utility of naturally occurring restriction fragment length polymorphisms (RFLPs) as genetic markers was first demonstrated when such RFLPs were used to map the DNA sequences coding for particular tRNA molecules in yeast (Olson et al. 1979). Since that time, RFLP mapping has become an increasingly important tool for the molecular genetic analysis of complex genomes, most notably the human genome (Drayna and White 1985). But, in many cases, the applicability of the method has been limited severely by the small number of polymorphisms that can be detected between closely related genomes using the available unique-sequence probes. In a few cases, such as fine-structure mapping of

portions of the human genome, repetitive-sequence elements have been used as probes to detect numerous polymorphisms at once, thereby extending the applicability of the method (Gusella et al. 1982; Law et al. 1982). Nevertheless, most RFLP-mapping studies continue to be based exclusively on the use of unique-sequence probes. For example, although it has been known for years that the category of human repetitive-sequence elements known as "minisatellite," or "variable number of tandem repeat" (VNTR), elements are sufficiently variable that they can be used in DNA fingerprinting studies for unambiguous identification of individuals (Jeffreys et al. 1985), development of cloned probes specific for individual VNTR loci was considered to be a logical preliminary to the use of VNTR elements in mapping (Nakamura et al. 1987).

Here we show that under appropriate conditions repetitive-sequence probes can be used to identify quickly large numbers of RFLPs that appear widely dispersed throughout the *V. carteri* genome and that most such RFLPs exhibit the meiotic stability and Mendelian segregation patterns required for useful genetic markers. Two (apparently allelic) pairs of these RFLPs were found to cosegregate with alleles at the *regA* locus. These are the first markers that have been shown to be linked to *regA* and may provide the starting place for a chromosome walk designed to clone the locus. We believe that the strategy we have developed for using repetitive-element probes for RFLP mapping may have widespread applicability in developmental genetics.

Results

Preliminary observations used to select probes and enzymes for use

The two most divergent *Volvox* strains available to us at the time that this study began were HK 10 and 69-1b, which is an F₁ male from an HK 10 × HK 9 cross. Because the two parental strains were isolated from the same pond, and because 69-1b probably retains only about half of the genetic differences that distinguished HK 9 from HK 10, we did not expect to observe a great deal of polymorphism between HK 10 and 69-1b. This suspicion was supported by pilot studies in which no RFLPs were detected using the unique-sequence probes then available to us and a variety of restriction enzymes. We therefore chose to examine probes that would detect more genomic sequences and ones with a greater probability of exhibiting genetic variation (such as simple sequence DNA, or fold-back repetitive elements). Six repetitive-element probes, identified in Table 1 as EP, SX, 1A, 9BH, 10B, and V26, were developed as a consequence of that decision and were used here; some of their properties are given in Materials and methods.

Initial studies used genomic DNA digested with restriction enzymes having 6-base recognition sequences, such as *Bam*HI. With the exception of V26, all probes detected a continuous smear of hybridizing fragments on Southern blots of such preparations. In contrast, restriction enzymes with 4-base recognition sequences regu-

larly generated patterns containing many well-resolved bands of high molecular weight, plus a smear of unresolvable lower-molecular-weight fragments; such enzymes were therefore used in combination with all probes, except V26, to identify interstrain RFLPs. However, prolonged electrophoresis was used to run unresolved small fragments off the gel and provide maximum resolution of the remaining, longer fragments. The V26 probe differs from all the others in that it detects a single intensely hybridizing band and several weaker (apparently single-copy) bands with a *Bam*HI digest; therefore this probe was only used in conjunction with a *Bam*HI digest.

RFLP frequency

When the six probes listed above were used in 26 different enzyme/probe combinations (Table 1) to analyze DNA samples from two strains of interest (HB11A, *regA*⁻ female, and ADM, the standard, wild-type male strain), a total of 1232 bands were identified. Of these, 291 bands (or ~24%) were present in DNA from only one of the strains and were therefore identified as interstrain RFLPs. Polymorphism frequencies detected with individual enzyme/probe combinations (excluding *Bam*HI/V26, which is a special case, as noted) ranged from 10% to 33% (Table 1).

Volvox is haploid, and alleles exhibit a mutually exclusive relationship (Huskey et al. 1979a). However,allelism is not readily established in the present analysis. Because the repetitive-element probes being used here detect numerous restriction fragments in each digest—most of which are run off the gel and excluded from the analysis—we consider it imprudent to assume either that comigrating fragments in two strains are identical, or that a pair of fragments exhibiting differential presence in the two strains are allelic. As a consequence, we have adopted the following conventions: (1) Each visible band is scored as a separate character; thus, bands of identical mobility in any two strains are scored as two characters. (2) Each band present in only one of the two strains is scored as a separate polymorphism. Although the second convention tends to overestimate the number of differences between strains, this should be more than compensated for by the first convention, which overestimates the number of characters being compared. As a result, the polymorphism frequencies reported in Table 1 are only approximations and probably represent minimum estimates.

To estimate what fraction of the above-mentioned RFLPs might be due to interstrain differences in DNA methylation patterns, samples of genomic DNA from selected male and female strains were digested in parallel with restriction enzyme isoschizomers that exhibit differential sensitivity to methylation of a common recognition sequence. When these restriction digests were compared on Southern blots, using four different hybridization probes, fewer than 5% of the strain-specific bands exhibited differential cutting by the methylation isoschizomers (data not shown). Thus, the vast majority

Table 1. Polymorphism frequencies with 26 enzyme/probe combinations

Probe	Enzyme	Number of bands ^a	Percent polymorphic ^b	Probe	Enzyme	Number of bands ^a	Percent polymorphic ^b
EP	<i>Alu</i>	44	20	9BH	<i>Alu</i>	33	7
	<i>Hae</i>	56	30		<i>Hae</i>	39	33
	<i>Hha</i>	53	28		<i>Hha</i>	24	33
	<i>Hinf</i>	47	19		<i>ScrI</i>	41	27
	<i>Msp</i>	43	23		<i>Taq</i>	59	10
	<i>Rsa</i>	56	16	Average polymorphism with 9BH:			22
	<i>Sau</i>	51	18	10B	<i>Hae</i>	47	10
	<i>ScrI</i>	54	33		<i>Hinf</i>	68	15
	<i>Taq</i>	54	13		<i>Msp</i>	43	23
Average polymorphism with EP:			22		<i>Rsa</i>	52	10
SX	<i>Hinf</i>	56	23		<i>Sau</i>	54	26
	<i>Msp</i>	50	30	Average polymorphism with 10B:			16
Average polymorphism with SX:			27	V26	<i>Taq</i>	49	12
1A	<i>Msp</i>	40	30		<i>Bam</i>	14	50
	<i>Rsa</i>	51	33	Grand totals:			1232
	<i>Taq</i>	54	27				24
Average polymorphism with 1A:			30				

^a Total number of bands detected in Southern blots of ADM plus HB11A DNA.^b Percent of all detected bands that were present in one strain but not the other.

of the strain-specific polymorphisms appear to have a basis other than interstrain differences in methylation patterns.

Mitotic stability of RFLPs

A survey initiated for other purposes provided data permitting estimation of the mitotic stability of the RFLPs described above. Genomic DNAs from nine bleomycin-induced *regA*⁻ strains and selected wild-type strains

were analyzed in an unsuccessful search for novel RFLPs resulting from bleomycin-induced mutations at the *regA* locus. These mutants had been derived from two different subcultures of HK 10, called HK 10A and EVE-2; at the time of analysis, EVE (a subclone of HK 10 and the progenitor of EVE-2) had been isolated from EVE-2 for about 1 year and from HK 10A for more than 5 years. The enzyme/probe combinations used to analyze these strains were the same as those used in the next section for segregation analysis (see Fig. 3).

Figure 1. Example of Southern blots used to analyze RFLP instability in various HK 10 lineages. DNA samples from three strains derived from HK 10A [namely, B1 (a), B2 (b), and B60 (c)], five strains derived from EVE-2 [namely, HB6 (d), EVE-2 (e), HB4 (f), HB15 (g), and HB19 (h)], and three "standard" strains [namely, EVE (E), ADM (A), and TOM-10 (T)] were digested with *RsaI* and electrophoresed in a 1% agarose gel. The resulting Southern blot was hybridized with probe 1A. Note that most bands are conserved among different HK 10A- and EVE-2-derived strains. The arrow points to a band seen in all five EVE-2-derived strains that is absent from both the HK 10A-derived strains and from EVE. Several differences are seen between ADM and TOM-10; these are all members of the group of putative *mt*-linked RFLPs discussed in the text.

On a sample Southern blot from this survey (Fig. 1), a band is seen in all four EVE-2 derived mutants, and in EVE-2 itself, which is not present either in EVE or any of the HK 10A-derived mutants. This apparently represents a RFLP that arose during the derivation of EVE-2 from EVE and was passed on to all of its asexually derived progeny strains. But, of 1232 bands resolved in this particular survey, only 12 bands were observed in which one or more of the EVE-2-derived strains differed from EVE; more than 99% of all bands were identical in all members of the lineage after 1 year of independent propagation and several rounds of mutagenesis and cloning. When the HK 10A-derived strains were compared with EVE, 56 bands (<5% of all bands resolved) differed between EVE and one or more (usually all) of the HK 10A-derived strains. Thus, after more than 5 years ($\sim 10^3$ asexual generations) of isolation, more than 95% of all detected bands were shared by all strains tested.

Segregation analysis

Although many of the 291 RFLPs initially identified were bands that hybridized too weakly or were not resolved clearly enough to provide reliable markers for segregation analysis, each enzyme/probe combination yielded an average of five bands that both distinguished HB11A from ADM and were sufficiently strong and clear to be highly suited for segregation studies. Of the foregoing, 94 RFLPs (identified in Fig. 2) were selected for analysis of segregation patterns in 10–11 progeny from an HB11A $\times$ ADM cross. The segregation patterns for these 94 RFLPs fell into 36 groups (Fig. 3). Whereas a plus sign is used in Figure 2 to indicate which of the parents exhibited a particular band, in Figure 3, RFLPs that showed “allelic” patterns in parents and progeny (i.e., one was present only when the other was missing) have been assigned to the same segregation group.

Nearly half of the segregation groups (17 of 36) contained only one RFLP. Only four groups contained more than 4 RFLPs: Group 3, containing 13 RFLPs, was the largest, followed by group 17 with 8, group 8 with 7, and group 1 with 6. All group-3 RFLPs were detected with a single probe (EP), and all group-1 RFLPs were detected with another (V26). Groups 17 and 8, however, contained RFLPs detected by at least three different probes. Two segregation groups, 17 and 26, are of special interest because markers in them showed perfect concordance with alleles at two of the conventional loci examined in this same study, namely, the *mt* (mating-type) locus and the *regA* locus, respectively. No RFLPs were found that cosegregated with the *chl* and *mes* loci.

The eight RFLPs in group 17 were all cases in which a band was present in all females but absent in all males. The concordance was also seen in TOM-10, a female that had been derived by 10 successive generations of backcrossing to the standard male strain; this indicates that all eight group-17 markers cosegregated with *mtf* through 10 successive sexual generations.

Because of our interest in identifying RFLPs tightly linked to the *regA* locus, nine additional offspring, including seven from a second *regA*[−] $\times$ *regA*⁺ cross, were

analyzed to determine whether they also exhibited concordance between alleles at the *regA* locus and the Sau10B-1/Sau10B-3 RFLP pair of group 26 (Fig. 4). The presence or absence of these two bands in each strain was confirmed at least twice with independent enzyme digestions and Southern blots. In total, 19 out of 20 progeny showed concordance between the *regA*⁺ allele and the Sau10B-1 RFLP and between the *regA*[−] allele and the Sau10B-3 RFLP. When the second pair of group-26 RFLPs (HinfEP-4 and HinfEP-4a) was analyzed further in the same way, 18 out of 19 progeny showed the parental combinations of these two RFLPs with alleles at the *regA* locus. It is noteworthy that it was the same individual that provided the exception for both pairs of RFLPs, indicating that it may have undergone a relatively rare recombinational event.

Discussion

Use of repetitive-element probes for RFLP mapping

The probes used in this study to search for strain-specific RFLPs all detected moderately to highly repetitive sequences. Although repetitive-element probes have been used occasionally for RFLP mapping in the past (Gusella et al. 1982; Law et al. 1982), this constitutes a departure from usual procedures. It is generally assumed that the most useful probes for RFLP mapping are those that detect only one or a few homologous sequences in genomic DNA, as repetitive-element probes frequently generate excessively complex hybridization patterns that confound analysis. When we tested our repetitive-element probes on genomic DNA digested by restriction enzymes with 6-base recognition sequences, all but one probe generated smears of hybridization that were unintelligible. Five of these probes became useful for detecting discrete genomic fragments and strain-specific RFLPs only when they were employed with Southern blots in which DNA was first digested with enzymes having 4-base recognition sequences and then electrophoresed long enough to run most of the DNA off the gel. Under these conditions, only the longest members of each restriction-fragment population are examined. With 4-base cutters, the average size of the restriction fragments is expected to be 0.256 kb, but the RFLPs detected with our protocol ranged in size from 1.4 to 13 kb and averaged 6.0 kb (Fig. 2). Examination of this minor fraction of exceptionally long members of each restriction digest was based on the empirical observation that it worked; there is no reason to believe that these longer fragments should differ systematically from their shorter counterparts in the rate of accumulation of strain-specific differences per kilobase of DNA.

Use of the enzyme/probe combinations described here carries two disadvantages, relative to more conventional techniques of RFLP analysis. The first has been mentioned: Allelic relationships between RFLP pairs are more difficult to establish than when probes are used that detect only one or a few homologous restriction fragments per genome. For this reason, we designate each band seen in one strain but not another as a sepa-

Enz	Prb	I#	Grp	MW	F	M
Alu	EP	1	4	9.4	-	+
		4	11	3.6	+	-
Alu	9BH	1	8	4.8	+	-
		2	23	4.3	-	+
		3	30	3.8	-	+
		6	19	3.0	-	+
		10	35	1.4	+	-
Bam	V26	1	1	5.8	-	+
		2	1	4.8	+	-
		3	1	4.3	+	-
		4	2	3.1	-	+
		5	1	2.9	-	+
		6	1	2.5	+	-
		7	1	1.8	-	+
Hae	EP	1	3	10	+	-
		4	12	7.2	+	-
		9	13	2.4	-	+
		10	11	2.3	+	-
Hae	9BH	1	8	4.6	+	-
		3	24	2.4	-	+
		3a	17	2.1	+	-
Hae	10B	1	30	7.0	+	-
		2	31	4.7	+	-
		3	32	2.9	+	-
		4	8	2.3	+	-
Hha	EP	1	3	10.4	+	-
		2	3	9.3	+	-
		2a	3	9.0	-	+
		3	3	8.4	-	+
		4	4	8.0	-	+
		5	4	7.2	+	-
		6	5	6.4	+	-
Hha	9BH	7	5	6.3	-	+
		1	5	6.4	+	-
		1a	5	6.3	-	+
		2	8	5.3	+	-
		3	20	4.3	-	+
Hinf	EP	4	21	4.0	-	+
		4a	21	3.9	+	-
		1	29	9.6	+	-
		3	4	7.0	+	-
		4	26	4.2	+	-
Hinf	SX	4a	26	4.1	-	+
		5	34	3.8	-	+
		3	19	1.9	-	+
Hinf	10B	1	25	9.1	+	-
		3	36	4.8	+	-
Msp	EP	1	3	10	+	-
		1a	3	9.7	+	-
		2	3	8.7	+	-
		2a	3	8.5	-	+
		4	3	7.9	-	+
Msp	SX	3	10	3.5	+	-
		1	14	8.3	+	-
		1a	14	7	-	+
		2	15	5.3	+	-
Msp	1A	3	16	4.4	+	-
		1	33	6.3	+	-
		2	28	4.9	+	-
Msp	10B	2a	28	4.7	-	+
		3	17	3.9	+	-
		1	32	18	+	-
Rsa	EP	2	25	4.6	+	-
		3	19	3.2	-	+
Rsa	1A	1	3	10.5	+	-
		1	27	7.0	+	-
		2	28	6.7	+	-
		3	28	6.5	-	+
		4	17	4.8	+	-
		5	17	3.8	+	-
		6	15	3.5	+	-
Rsa	10B	7	17	3.2	+	-
		1	8	4.7	+	-
		2	17	3.2	+	-
Sau	EP	3	18	2.3	+	-
		1	6	13	+	-
Sau	10B	1	26	11	-	+
		3	26	9.8	+	-
		5	33	8.2	+	-
		6	25	7.9	+	-
Scrf	EP	1	3	9.6	+	-
		2	7	7.6	-	+
		3	8	6.4	-	+
Scrf	9BH	2	8	5.1	+	-
		3	23	3.9	-	+
Taq	EP	1	9	10	+	-
		2	3	7.9	-	+
		4	10	4.2	-	+
		5	10	4.1	+	-
		1	17	3.6	+	-
Taq	10B	1	17	2.8	+	-
		2	19	2.6	-	+

Figure 2. The 94 RFLPs used for segregation analysis. Each RFLP is identified by the enzyme (Enz) and probe (Prb) used to detect it, plus its identification number (I#). Thus, the first RFLP in the list is identified as *Alu*EP-1. For each RFLP, the segregation group (Grp) to which it belongs, its approximate size in kb (MW), and its presence (+) or absence (-) in the female (F) and male (M) parents (namely, HB11A and ADM, respectively) are indicated.

Mark	HB11	Adm	5	7	8	10	16	25	26	27	29	32	33	#R
mt	f	m	f	f	f	f	f	f	f	f	f	f	m	
reg	-	+	+	+	+	-	-	+	+	-	-	-	-	
mes	-	+	+	-	+	-	-	+	+	+	-	+	+	
chl	-	+	-	+	-	-	+	+	+	-	-	-	+	
1														6
2														1
3														13
4														4
5													NA	4
6														1
7														1
8														7
9														1
10														3
11														2
12														1
13											NA			1
14														3
15														2
16														2
17														8
18													NA	1
19														4
20													NA	1
21													NA	2
22														1
23														1
24														1
25														3
26														4
27					NA									1
28														4
29														1
30														2
31														1
32								NA						2
33														2
34														1
35													NA	1
36														1

Figure 3. Summary of the 40 segregation patterns observed for four conventional markers, plus all 36 groups of RFLPs among 11 progeny of an HB11A × ADM cross. Markers (Mark) analyzed included four conventional loci [*mt* (m = male; f = female), *regA*, *mes*, and *chl*], plus the 36 RFLP groups, members of which are defined in the legend to Fig. 2. Numbers at the tops of the fourth through fourteenth columns indicate the progeny numbers assigned to the individual offspring from the HB11A × ADM cross that were analyzed in this study. (#R) The total number of RFLPs in each segregation group. (NA) For technical reasons, data for that segregation group were not obtained for that individual. Segregation patterns are indicated by light vs. dark stippling of boxes; to determine whether dark stippling indicates presence or absence of any particular RFLP, the patterns for HB11A and ADM can be compared with the information given in the legend to Fig. 2, where these two strains are referred to as F and M, respectively, and the presence or absence of individual RFLPs for each strain is indicated.

rate marker unless or until independent evidence of allelism between bands is obtained. (However, as the number of progeny exhibiting such a mutually exclusive

relationship increases, so, of course, does the probability that the pair in question are truly allelic.) The second disadvantage is more long range: It complicates the use

Figure 4. Example of Southern blots indicating linkage between two RFLPs and the *regA* locus. *Sau*3a-digested DNA from HB11A, ADM, six *regA*⁻ progeny (108-10, -16, -27, -29, -32, and -33), and six *regA*⁺ progeny (108-5, -7, -8, -25, -26, and -2) were electrophoresed on a 0.75% agarose gel. The resulting Southern blot was hybridized with probe 10B. Note that RFLPs *Sau*10B-1 and *Sau*10B-3, marked by arrows 1 and 3, show perfect concordance with the pattern of segregation of the *regA*⁺ and *regA*⁻ alleles, respectively.

of linked RFLP markers as starting places for chromosome walks designed to clone loci of interest, because it necessitates cloning of the polymorphic fragment itself, and isolation of a unique subfragment from it, before the walk can be begun.

Outweighing those disadvantages, there is one significant advantage of using repetitive elements as RFLP probes: namely, the rate at which genetic markers can be identified and linkage data compiled per unit of effort. In our pilot study with several unique (or small multigene family) probes, we were unable to detect any strain-specific differences in any of the restriction digests of genomic DNA that we tested. The number of additional unique-sequence-probe/enzyme combinations that would have been necessary to define a significant number of useful RFLPs is indeterminate, but undoubtedly it could have been very large. In contrast, with only six repetitive-element probes, drawn more or less at random, we were able to define nearly 100 RFLPs that segregated reliably as Mendelian markers.

Frequency and mitotic stability of RFLPs

Using the 26 enzyme/probe combinations listed in Table 1, 1232 bands were detected in Southern blots of DNA from HB11A and ADM; 24% of these bands were identified as strain-specific RFLPs. Statistical analysis of the data in Table 1 suggests that there is no significant difference in average RFLP frequencies observed with the

different probes, and hence no evidence that these six probes detect classes of sequences that differ significantly in genomic stability. However, a class of sequences with significantly greater polymorphism and lower stability was detected, using a probe not described previously: Much higher RFLP frequencies (>50%) were observed when the synthetic polynucleotide poly(dGdT) · poly(dCdA) (abbreviated hereafter GT/CA) was used, but RFLPs detected with GT/CA were not included in data reported here because interstrain and segregation analyses indicated that they were too unstable to serve as reliable genetic markers. Because these studies indicated that tracts of GT/CA may be associated with hypervariable regions in *Volvox*, as has been reported previously for other organisms (Rich et al. 1984), the other six probes used here were tested for GT/CA homology. Three of them (EP, SX, and 9BH) exhibited weak cross-hybridization with GT/CA. However, detailed examination of RFLP patterns indicated clearly that, despite the weak cross-hybridization of these three probes with GT/CA, few if any of the genomic fragments that any of them detected were fragments detected by GT/CA itself. Nevertheless, the fact that mitotic instability had been observed for elements detected with GT/CA strongly suggested that the mitotic stability of genomic elements detected with the other probes should be examined more carefully.

To estimate mitotic stability, we used all of the en-

zyme/probe combinations listed in Table 1 to compare the patterns generated from EVE, our “standard” clonal isolate of HK 10, to those generated from other strains that had been derived from HK 10. Not only had all of these strains been propagated independently for a considerable period, many had been mutagenized and cloned several times during their derivation. At the time of analysis, EVE had been separated from EVE-2 and its derivatives for about 200 asexual generations, and from HK 10A and its derivatives for more than 1000 asexual generations. Approximately 1% and 5% of the bands were different in these two sets of comparisons, respectively. Therefore, we estimate the rate at which new variations accumulate in fragments homologous to the repetitive sequences used here to be not more than 1% per 200 asexual generations. [Note: Because the production of a new cohort of reproductive cells during asexual embryogenesis requires eight successive mitotic divisions (Green and Kirk 1981), the frequency with which new variants is generated is nearly an order of magnitude less if expressed in terms of mitotic cycles rather than asexual generations.]

Even at this low rate of change, however, because one asexual generation is completed every 2 days under optimum culture conditions, novel RFLPs could accumulate in serially propagated cultures at nearly 1% per year, and the RFLP map would change considerably in cultures maintained by serial transfer over several years. Therefore, to preserve the germ plasm of ADM and EVE in a form as intact as possible, we have performed backcrosses to generate a male (DRG-11) and female (TOM-10) that are congenic in RFLP patterns with EVE and ADM, respectively (except for RFLPs tightly linked to the *mt* locus), and have frozen zygotes from crosses of EVE $\times$ DRG-11 and TOM-10 $\times$ ADM.

Meiotic stability of the RFLPs

Meiotic stability is also required to establish RFLPs as useful markers for genetic mapping. Three types of observations made here indicate relatively high meiotic stability of all RFLPs listed in Figure 2. Specifically: (1) all of these RFLPs appeared to segregate as simple Mendelian markers within the limits of precision to be expected with such a restricted sample size. (2) New or modified markers were detected in the progeny at only a very low frequency. Specifically, only two bands were detected among the progeny that were different from any detected in the parents. Because 10 progeny were examined with all of the enzyme/probe combinations that were used to resolve 1232 bands in the parents, this yields an estimate of the maximum rate of meiotic variation of less than 0.02%. (3) The strongest evidence to date for meiotic stability was given by the observation that all eight of the group-17 RFLPs that had been detected in EVE using several different probes—but none of the other RFLPs that distinguish EVE from ADM—were present in TOM-10, the tenth-generation female derived from EVE by repetitive backcrossing to ADM. Thus, these eight RFLPs cosegregated with one another

—and with the *mtf* allele—without detectable change, through 10 successive sexual generations.

Some meiotic variation resulting from recombination and/or gene conversion is to be expected, of course, but the evidence given above clearly indicates that the RFLPs detected here possess sufficient meiotic stability to be suitable for long-term mapping studies.

Linkage relationships among RFLPs

To be employed as markers for development of a useful genetic map, it is also essential that the RFLPs be distributed widely throughout the genome and yet exhibit consistent linkage relationships with one another and with conventional markers. A highly clustered organization would be expected to produce a small number of segregation groups with many RFLPs in each group. In contrast, we observed that the 94 RFLPs studied exhibited 36 different segregation patterns, with a small number of RFLPs in most groups. Of the 36 groups, only 4 contained a sizeable number of RFLPs (6–13), 17 groups contained only a single RFLP, and the remaining 15 groups contained only 2–4 RFLPs. Many of the latter groups contain RFLP pairs that are probably allelic, as they show a mutually exclusive distribution pattern. But because we had no independent method to demonstrate allelism and scored each strain-specific band as an independent marker, a single chromosomal rearrangement could result in changes that would be scored in our analysis as up to four separate RFLPs. Therefore, our data may actually overestimate the degree of clustering of RFLPs.

The larger number of RFLPs associated with four of the segregation groups may be the result of (1) a tight cluster of similar polymorphic sequences homologous to one of the probes, (2) a single polymorphic region of DNA that is detected by several different enzyme/probe combinations and is therefore scored as multiple, independent RFLPs, or (3) a large chromosomal region with a low frequency of recombination. Tight clustering of similar polymorphic sequences is probably the best explanation to account for the 13 RFLPs in group 3 (all detected with the EP probe) and the 6 in group 1 (all detected with the V26 probe). However, the other two large RFLP groups, groups 8 and 17, contain representatives detected with at least three different probes. Since these probes show very limited cross-hybridization, and only occasionally detect RFLPs together in any of the other 34 segregation groups, they are probably identifying several discrete, but closely linked, markers.

In short, our probes appear to detect polymorphisms that are widely distributed and should ultimately provide markers in many regions of the genome.

Linkage to conventional markers

The two RFLP groups of greatest interest in this initial survey were groups 17 and 26, because they exhibited cosegregation—presumptive evidence of linkage—with alleles at the *mt* locus and the *regA* locus, respectively.

The evidence indicating strongly that RFLPs in group 17 are linked to the *mt* locus is discussed above. The

large number and tight linkage of RFLPs in this group are consistent with the genetic map developed by Huskey et al. (1979a) in which the second most highly represented linkage group, and the one with the most tightly linked markers, was the one containing the *mt* locus. In the closely related alga *Chlamydomonas reinhardtii*, the *mt* locus appears to lie in a region of suppressed recombination (Gillham 1969). We therefore favor the hypothesis that the abundance of RFLPs tightly linked to the *mt* locus is a reflection of low recombination frequency in this chromosomal region.

The RFLPs cosegregating with the *regA* locus in progeny from the HB11A × ADM cross are of particular interest. From the combined results of two crosses, using two different *regA* mutants as the female parents, the parental combinations of *regA* alleles and RFLPs were observed in 19 out of 20 progeny analyzed for the Sau10B RFLP pair and in 18 out of 19 progeny analyzed for the HinfEP RFLP pair. (It is noteworthy that the same female exhibited the nonparental pattern for both RFLP markers.) Linkage of these RFLPs to the *regA* locus is highly significant by a chi-square test. These data suggest that the Sau10B-1/Sau10B-3 and HinfEP-4/HinfEP-4a RFLPs are two allelic pairs, both located about 5 map units from the *regA* locus. These two RFLP pairs are the first markers of any kind to be found linked to the *regA* locus (Huskey and Griffin 1979; Huskey et al. 1979a; R. Huskey, pers. comm.; K. Huson, J. Harper, and D. Kirk, unpubl.). The reasons for our particular interest in the *regA* locus are outlined in the introduction. The identification of RFLPs linked to this locus opens up the possibility of cloning it by chromosome walking or jumping (Collins and Weissman 1984).

Although it is difficult to estimate the number of possible segregation groups to be expected in an analysis like the one described here, the fact that we failed to identify RFLP markers linked to two of the four conventional markers examined suggests that it is likely that many portions of the genome remain unmarked by the 94 RFLPs examined so far. Since the studies reported here were performed, Richard Starr has provided us with a second pair of *V. carteri* isolates (from India) that are interfertile with the strains employed here but greatly increase the number of RFLPs available for linkage analysis. Expansion of the RFLP map of the species by analysis of progeny derived from crosses between these two sets of isolates is underway.

The strategy reported here for identifying RFLPs linked to genes of interest by using repetitive-sequence probes to examine rapidly many regions of the genome should, in principle, be adaptable to other organisms. We anticipate that this strategy will prove particularly useful in instances where, as with *Volvox*, the available levels of genetic diversity (or even time and money) preclude development of a detailed RFLP map based on unique-sequence probes. Another adaptation of this strategy that should also be broadly applicable is for DNA fingerprinting within species that are not as amenable to fingerprinting analysis by use of the human VNTR probes as mice are (Jeffreys et al. 1987). For ex-

ample, a strategy based on the one reported here for identification of RFLPs (use of 4-base cutters, prolonged electrophoresis, and repetitive-element probes) has been used successfully for DNA fingerprinting of soybean cultivars that had exhibited minimal genetic diversity by conventional RFLP methods (G. Lark, pers. commun.). In *Volvox*, a single probe of the type used here is capable of detecting markers that lend themselves to either DNA fingerprinting or gene mapping; we suspect that this will frequently prove to be the case in other species as well.

Material and methods

Volvox strains

All strains used here were derived from cultures of *V. carteri* f. *nagariensis*, strains HK 10 (UTEX 1885), and/or 69-1b (UTEX 1886), provided at various times by the University of Texas Culture Collection of Algae, where they have been maintained by asexual reproduction and serial transfer since their isolation in 1967 and 1969, respectively. Strains HK 9 (male) and HK 10 (female) were the original strains isolated in 1966–1967 from a pond in Japan (Starr 1969); subsequently, 69-1b, an F₁ male from an HK 10 × HK 9 cross, replaced HK 9 as the standard laboratory male strain. HK 10A is a subculture of HK 10 that was propagated in this laboratory by serial transfer for more than 1000 generations. When initial RFLP studies revealed variation within and among various subcultures of HK 10, subclones were established from individuals drawn at random from 69-1b and HK 10 cultures that had been obtained from the Culture Collection of Algae in December 1982. Those subclones, designated ADM and EVE, respectively, became the reference standard for RFLP studies reported here. Strains TOM-5 and TOM-10 are fifth- and tenth-generation females (respectively) derived by crossing EVE × ADM and then backcrossing female offspring from each successive generation to ADM; strain DRG-11 is an eleventh-generation male derived similarly by sequentially backcrossing male offspring to EVE. Thus, TOM-10 and DRG-11 constitute “congenic female” and “congenic male” strains, respectively, each of which should contain more than 99% of the genetic background of the progenitor strain of opposite mating type, except for loci tightly linked to mating type. Strain SER-3 is a somatic regenerator (*regA*[−]) mutant derived spontaneously from EVE. Strain EVE-2 is a chlorate-resistant (Chl^R) and methionine sulfoximine-resistant (Mes^R) strain derived from EVE by two successive rounds of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis and selection. HB strains are *regA*[−] derivatives of EVE-2 induced by bleomycin, a chromosome-rearranging agent (Vig and Lewis 1978). Strains 138-2 through 138-33 are individual progeny from a cross (138) between HB11A × ADM; strains 108-1 through 108-24 are progeny derived from a cross (108) between SER-3 × ADM. Because isolation of DNA from male strains is complicated by the fact that dense cultures of males tend to go sexual and self-destruct, the preliminary progeny analyses reported here were deliberately biased heavily in favor of female progeny.

Culture conditions

Cultures were maintained in an asexually reproducing state in standard *Volvox* medium (SVM) (Kirk and Kirk 1983). Stock cultures were maintained in tubes under slow growth conditions. For DNA isolation, female strains were grown to sta-

tionary phase under conditions described previously (Kirk and Kirk 1983), but male cultures were grown at 28°C (rather than 32°C) and were harvested during exponential growth.

Mating

Strains to be mated were grown synchronously on a 48-hr asexual life cycle under conditions similar to those described above, except that a 24-hr illumination cycle (16L : 8D) and an incubation temperature of 28°C were employed to achieve maximum fertility (J. Zeikus, pers. commun.). After mating, cultures were maintained under this same regimen until germ-lings were isolated.

Sexual development was triggered by addition of inducer (Starr and Jaenicke 1974) to synchronous, asexual cultures containing juveniles in the early expansion stage. Female cultures were covered with a white plastic garbage bag 48 hr later to reduce illumination and to slow development so that males and females would reach maximum fertility at the same time (J. Zeikus, pers. commun.). Settled organisms were pipetted off the bottom of culture flasks 72 hr postinduction (when males had begun to release sperm but egg-bearing females had not yet hatched); 2 ml of such a suspension of females was passed through a drawn pipette to release the egg-bearing juveniles into a sterile glass petri dish (100 × 20 mm), and 4 ml of males and 15 ml of medium from the male culture were added. Medium (15–20 ml) from the male culture was added to the dish 24 hr later. After zygotes had matured for 2 weeks, they were manually separated from vegetative materials and debris, washed six times with SVM, and incubated in a covered depression slide in SVM. When zygotes began to hatch after 3 days, germ-lings were cloned into multiwell plates containing fresh SVM. When zygotes were to be frozen, washing was omitted, the medium was brought to 5% methanol, and 1.5-ml aliquots of zygote suspensions were frozen in Nunc cryo tubes at –20°C and transferred to –80°C 24 hr later.

Mutagenesis

Early cleavage-stage embryos were broken out of parental spheroids and suspended in SVM that had been supplemented with 10 times the usual concentration of HEPES buffer and adjusted to pH 8.0 at 32°C. Then 0.25 µg/ml MNNG, or 100–300 µg/ml bleomycin sulfate, was added and cultures were incubated in the dark for at least 3 hr. This MNNG treatment results in about 50% lethality. The lethality of bleomycin treatment is much more variable because of a precipitous decline in toxicity during early cleavage (Kirk et al. 1987); bleomycin-induced Reg mutants studied here were isolated in experiments in which 40–90% lethality was observed. *Chl^R* mutants were selected 24 hr postmutagenesis in liquid SVM containing 1.4 mM chlorate (Huskey et al. 1979b). For identification of Reg and *Mes^R* mutants, organisms were plated in a thin layer of 0.375% SeaPlaque agarose (FMC Corp.) in SVM over a 1% Difco Bacto-Agar/SVM base. For *Mes^R* selection, methionine sulfoximine was added to the agar base to a final concentration of 42 mM. Reg mutants were identified by visual screening.

DNA isolation

Isolation of nucleic acids from *Volvox* is complicated by the sulfated glycoproteins and polysaccharides that abound in the *Volvox* extracellular matrix, copurify with nucleic acids, and inhibit many nucleic acid-metabolizing enzymes. Of more than 100 variants of published DNA isolation methods tested, the following procedure is the only one that, in our hands, has rou-

tinely yielded *Volvox* genomic DNA satisfactory for Southern blot analysis.

Spheroids were collected by filtration, suspended in SVM, and broken with a loose pestle in a Dounce homogenizer to release mucilaginous extracellular matrix. Cells were pelleted by centrifugation in a Sorvall SS-34 rotor at 7000 rpm at 20°C for 5 min. (Except where noted, all subsequent centrifugations were carried out in the SS-34 rotor at 20°C and 10,000 rpm.) The supernatant and a translucent layer were removed, and the green pellet resuspended in 1 volume of 0.5M EDTA (pH 8.0), 1% 2-mercaptoethanol, and 4% sarcosine and immediately frozen, drop-wise, in liquid nitrogen. The frozen suspension and approximately 0.25 volume of dry ice were ground to a powder in a prechilled Moulinex coffee grinder; 0.1 volume of 10% SDS was then added, and the mixture was warmed to 42°C for 5–10 min. The solution was mixed with 1 volume of butanol, containing 1% cetyltrimethylammonium bromide (CTAB), and centrifuged for 10 min. The aqueous bottom layer was reextracted with butanol/CTAB until very little interface remained, and combined with 1 volume of H₂O, and the DNA was precipitated with (relative to the new volume) 0.1 volume of 4 M ammonium acetate and 0.7 volume of isopropanol (overnight at 4°C). The DNA was pelleted by centrifugation for 5 min, and the pellet dried and redissolved in 100 mM Tris-HCl (pH 8.0) and 50 mM EDTA. This solution was brought to 0.5% sarcosine and 0.2 mg/ml ethidium bromide; and 0.9 g of CsCl was added per gram of solution. The mixture was clarified by centrifugation for 10 min and centrifuged to equilibrium at 40,000 rpm in a Spinco model L2, using either an SW50.1 or type-50 rotor. Visible DNA bands were removed, and the ethidium bromide was extracted with isopropanol saturated with 3 M NaCl, 0.3 M Na citrate, at pH 7.0. DNA was precipitated at –20°C for at least 1 hr after adding 3 volumes of H₂O and (relative to the new volume) 0.1 volume 4 M ammonium acetate and 0.8 volume isopropanol. DNA was pelleted by centrifugation at 4°C for 10 min, washed twice with 70% ethanol, dried, and redissolved in 300 µl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 100 mM NaCl. Then 15 µl of 200 mM spermine-4-HCl was added, and the precipitated DNA pelleted by centrifugation in a microfuge. [Note: We now obtain a higher yield by decreasing the NaCl in the dissolving buffer to 10 mM and adding twice as much spermine.] The pellet was redissolved in 75 µl of 4 M ammonium acetate and 50 µl of 100 mM EDTA, reprecipitated with 0.8 volume isopropanol, pelleted, and redissolved in 1 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA.

Southern blotting and hybridizations

Preparation, electrophoresis, and blotting of restriction fragments, nick-translation of probes, hybridization and posthybridization washing of blots (final washing in 0.3 × SSC at 68°C for 30 min), and autoradiography were all performed according to standard procedures (Maniatis et al. 1982). Restriction digests were electrophoresed about 1.5 times as long as was required to run the dye front off the gel.

Hybridization probes

Six middle-repetitive DNA sequences from *Volvox* were used as hybridization probes. Two were “C-rich” probes: a 570-bp insert in pVGC3EP and a 650-bp *SalI*–*XhoI* fragment from pVGC3, abbreviated hereafter as EP and SX, respectively; these have been sequenced and described elsewhere (Harper 1986). VFB10B and VFB1A were λ Charon 30 genomic clones that were isolated because of their strong hybridization to a middle-repetitive “fold-back” fraction of the *Volvox* genome. [The fold-back fraction was isolated by heat-denaturing genomic DNA, quick

chilling on ice, and immediately digesting with S1 nuclease. Approximately 10% of the starting material survived the S1 treatment, was nick-translated, and used to probe a *Volvox* genomic bank (Harper 1986). The *Bam*HI inserts from VFB10B and VFB1A that contained the strongest homology to the fold-back fraction were subcloned into the polylinker of pUC8. The plasmid clones pVFB10B1-2 and pVFB1A1-2 used in this study, and abbreviated hereafter as 10B and 1A, have approximately 5- and 4.5-kb inserts, respectively. pVBT9BH, abbreviated hereafter as 9BH, was a genomic clone with a 2.7-kb insert containing a portion of a β -tubulin gene (Harper 1986); it was used here because it also contains a region exhibiting strong hybridization to the fold-back fraction. The sixth probe, V26, is a cDNA clone homologous to an abundant low-molecular-weight *Volvox* transcript of unknown nature. Probes EP and 9BH exhibit a moderate level of cross-hybridization to each other but detect different families of genomic fragments; the other probes exhibit no significant cross-hybridization under conditions used here.

RFLP identification

RFLPs were named according to the restriction enzyme and probe used for their detection; those detected by each enzyme/probe combination were then numbered in order of decreasing size. Enzymes used were: *Alu*I, *Bam*HI, *Hae*III, *Hha*I, *Hin*FI, *Msp*I, *Rsa*I, *Sac*II, *Sau*3A, and *Taq*I (obtained at various times from Bethesda Research Labs, Boehringer-Mannheim, New England Biolabs, and Promega Biotech and used according to the vendors' recommendations); numerical suffixes are omitted in RFLP names. Only the upper regions of each Southern blot, where individual bands could be resolved clearly, were used for counting bands and identifying polymorphisms. Bands were detected that ranged in signal strength from very weak to very strong on a single Southern blot; bands were only included in the analysis if they could be seen reproducibly in two or more strains. Restriction-fragment differences between ADM and EVE were generally confirmed by examination of TOM-5 and TOM-10 ("ADM-like" female strains) and several EVE-derived strains. A small number of reproducible intensity polymorphisms were detected; for simplicity, these are grouped with the much greater number of length polymorphisms. Restriction fragment length estimates were based on comparisons to *Hin*diIII fragments of λ DNA that were run on every gel, stained with ethidium bromide, and photographed with UV illumination.

Except where noted (for the markers in group 26, which are the focus of this paper) segregation analysis of RFLPs was done only once with only 11 progeny. Most of the RFLP-segregation data are presented here as the results of a preliminary survey and are not intended to constitute a definitive genetic analysis.

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Harper et al.

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Use of repetitive sequences to identify DNA polymorphisms linked to *regA*, a developmentally important locus in *Volvox*.

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