Identification of DNA Segments Capable of Rescuing a Non-Mendelian Mutant in Paramecium

Chung Sook Kim, John R. Preer, Jr., and Barry Polisky

Program in Molecular, Cellular, and Developmental Biology, Department of Biology, Indiana University, Bloomington, Indiana 47405

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ABSTRACT

The non-Mendelian mutant d48 of *Paramecium tetraurelia* contains micronuclear wild type *A* genes, but at autogamy and conjugation proper processing fails and new macronuclei lack *A* genes. When cloned *A* genes are injected into the macronucleus of d48, proper processing is restored at the next autogamy; d48 is rescued, becoming permanently wild type. In the present study we have injected portions of the *A* gene into d48. We find that the ability to rescue extends over a large portion of the gene, with highest activity near a series of 221-bp repeat units in the middle of the gene. Regions outside the *A* gene are inactive.

IN *Paramecium tetraurelia* the *A* gene, which codes for the surface immobilization antigen A, is located near the end of a macronuclear chromosome (FORNEY and BLACKBURN 1988). The non-Mendelian d48 mutant has a normal micronuclear *A* gene but normal processing fails at autogamy and conjugation, and the end of the macronuclear chromosome that bears the *A* gene, along with the *A* gene itself, is deleted (EPSTEIN and FORNEY 1984; FORNEY and BLACKBURN 1988). Since the *A* gene is missing, the A serotype cannot be expressed in d48.

Injection of virtually any DNA into Paramecium results in transformation (GODISKA *et al.* 1987; GILLEY *et al.* 1988; KIM *et al.* 1992). The DNA acquires telomeres and replicates autonomously. If serotype genes are injected they are expressed under proper environmental conditions. The DNA is maintained in the macronucleus until the next autogamy when the old macronucleus is replaced.

Injection of the whole *A* gene, or even incomplete fragments of the *A* gene, into the macronucleus of the d48 mutant results in transformation, *i.e.*, autonomous replication of the injected DNA. However, at the next autogamy, although the autonomously replicating DNA is lost, DNA processing occurs properly and the *A* gene reappears permanently, a phenomenon we refer to as "rescue" (KOIZUMI and KOBAYASHI 1989; YOU *et al.* 1991; JESSOP-MURRAY *et al.* 1991). Rescue is specific, for YOU *et al.* (1991, 1993) showed that the *B* gene as well as foreign DNAs cannot rescue. Moreover we show here that the closely related *G* gene of *P. primaurelia* as well as regions flanking the *A* gene do not rescue.

In the case of d48, the absence of *A* genes in the old macronucleus determines that the new macronucleus will also lack them, and the trait is inherited. Since genetic information is passed from old macronuclei to new macronuclei, MEYER (1992) has used the term "macronuclear inheritance" for this and a number of other cases of non-Mendelian inheritance in ciliates. The unique and totally unknown mechanism by which a gene in one nucleus can affect the DNA of the same gene in another nucleus is the main point of interest in this study. It is the purpose of our experiments to determine what region or regions of the *A* gene are effective in rescue.

MATERIALS AND METHODS

Strains, culture and serotypes: Strain d48 was derived from stock 51 of *P. tetraurelia* by EPSTEIN and FORNEY (1984). Paramecia were cultured as previously described (PREER *et al.* 1992). We are grateful to F. CARON for providing us with 156G antiserum. Serum tests were carried out in the usual way (SONNEBORN 1950).

Plasmids: The plasmid pSA14SB (GODISKA *et al.* 1987) contains the whole of the *A* gene from base −1590 to 11825 in a pT7T3–18 vector. Translation starts at +1 and ends at +8152. The 156*G* gene from *P. primaurelia* was a generous gift from F. CARON. It is designated pXΔ3 and includes the whole of the 156*G* gene of *P. primaurelia*. The other subcloned fragments depicted in Figure 1 were derived from pSA14SB except where indicated.

The following were subcloned using the indicated restriction sites into the plasmid pT7T3-18: pSA1.5 (−1131 *Eco*RI to 453 *Hga*I), pSA5.4 (13 *Xmn*I to 5464 *Ssp*I), pSA2.5 (2971 *Bgl*II to 5464 *Ssp*I), pSA3.0 (13 *Xmn*I to 2971 *Bgl*II), pSA3.9 (3175 *Hga*I to 7026 *Eco*RI) and pSA4.8 (7026 *Eco*RI to *Eco*RI 11825).

pSA2.4 and pSA1.56 were derived from pSA3.9 by removing portions of sequences. Thus pSA3.9 was digested with *Pst*I and the fragment containing 2371 bp (4655 *Pst*I to 7026 *Eco*RI) of the *A* gene and 2221 bp of the vector pT7T3-18 was isolated. It was then religated to produce pSA2.4. Likewise pSA1.56 was prepared by digesting pSA3.9 with *Ssp*I, isolating the fragment containing 1562 bp (5464 *Ssp*I to 7026 *Eco*RI) and 1938 bp of pT7T3-18, and religating at the *Ssp*I site.

pSA4.5 and its 5′ deletion derivatives were prepared by DAVID GILLEY in our laboratory. pSA4.5 was made by subcloning a 4.5-kb (13 *Xmn*I to 4517 *Pst*I) fragment into *Eco*RI/*Hinc*II digested pBluescript II KS+. The deletion series, pSA3.4

pSA2.1, pSA1.6, pSA1.0 and pSA0.3 was derived from *Bst*XI/*Eco*RI digested pSA4.5 by treating with exonuclease III for varying lengths of time. The fragments were then treated with mung bean nuclease and religated.

pSARI (3533–3750) DNA fragment includes most of the first repeat region of the *A* gene (3547–3767). It was prepared by means of polymerase chain reaction (PCR) using pSA1.0 as template and appropriate synthetic oligonucleotides as primers. The fragment was then cloned into the *Sma*I site of pBluescriptII KS+ to produce pSARI. pSGR1 was prepared in a similar manner: the 236-bp (3404–3639) fragment of the 156*G* gene sequence which included its first repeat region (3418–3639) was cloned into the *Sma*I site of pBluescript II KS+.

Microinjection: d48 cells were prepared for microinjection and injected as previously described (JESSOP-MURRAY *et al.* 1991). The plasmid DNAs were linearized at the *Xmn* I site in the vectors pT7T3–18 and pBluescript II KS+. The plasmids were coinjected with the 156*G* gene clone, pXΔ3, linearized at the *Xba* I site of the vector. The injection mixtures consisted of 1.3 mg/ml of the test DNAs and 1.0 mg/ml of the 156*G* gene DNA. The injected cells, generally about 100 cells for each test DNA, were isolated into depression slides and placed at 24°, the optimum temperature for expression of serotype 156G. After 2 days 4–10 cells from each depression were treated with anti-156G serum. The clones immobilized by the antiserum were transferred to test tubes, cultured at maximum fission rate (3–4 fissions per day) at 27°. After 20–30 fissions the cells were starved for 3 days in order to induce autogamy. Approximately 50% of the cells in each clone showed autogamy at this time. Cultures were continued at maximum rate for a few more days and again starved in order that all cells would have gone through autogamy at least once. The cells were then transferred to 34° and cultured at maximum rate. Aliquots from each tube were tested with anti-A serum after approximately two weeks at 34°.

RESULTS

Experiments: Our aim was to measure the efficiency of different fragments of the *A* gene to rescue, *i.e.*, to render d48 permanently wild type after the first autogamy following injection. Since injected DNA ordinarily is transformed into only about 10–30% of injected cells, a marker for transformation was used. Along with the plasmid containing the fragment of the *A* gene we coinjected the plasmid containing the *G* gene from *P. primaurelia*. Those cells transforming to serotype G 6–8 fissions after injection and before autogamy were assumed to be successfully injected and to have received both the *G* gene marker and the fragment of the *A* gene.

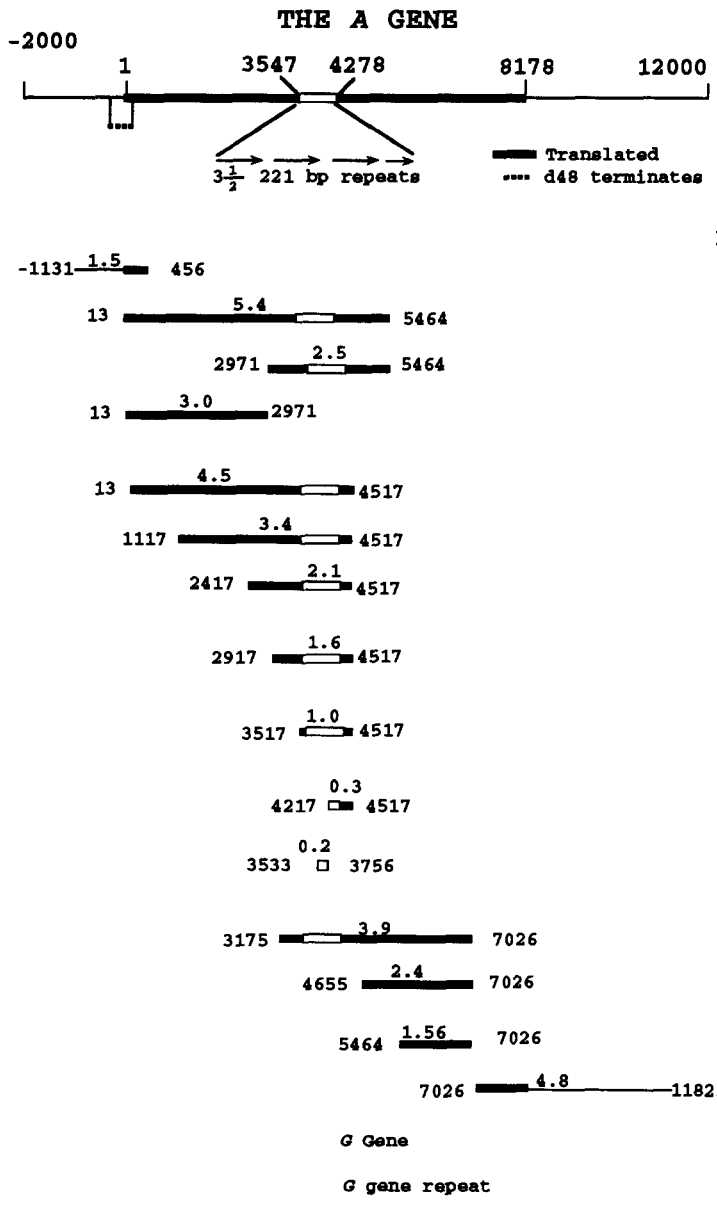
After autogamy, clones that had shown serotype G were placed under serotype A-inducing conditions and then tested for serotype A to measure the efficiency of rescue. The presence of serotype A cells in the clones after autogamy provided evidence that the micronuclear *A* genes of d48 had been processed properly at autogamy, *i.e.*, that rescue had occurred. Subclones were produced by isolating cells from clones that showed less than 100% serotype A. The subclones fell into two classes: some were near 100% serotype A, while others showed 0% serotype A. Except for an occasional 0% subclone that later produced some serotype A, all subclones underwent no further changes. We conclude that usually when a transformed clone containing portions of the *A* gene undergoes autogamy some of the cells are rescued and others are not. Thus the percentage of cells rescued (or the percentage of cells showing serotype A) after autogamy provides a measure of the effectiveness of rescue. The mean percent of serotype A cells in all clones injected with a particular DNA was computed as the best overall measure of the ability of that DNA to rescue. It was found that the *G* gene when injected alone showed no more rescue than the background frequency that could be attributed to spontaneous reversion of d48 (less than 1% in our experiments).

Upstream and downstream regions of the *A* gene do not rescue: The results of all the microinjections are given in Figure 1. It can be seen from the first line of data in the figure that the insert within pSA 1.5 starts at base −1131 upstream of the start of translation and ends at base 456 after the start of translation. In this experiment of about 100 cells that were injected 13 showed serotype G before autogamy. Of these, 12 showed 0% serotype A after autogamy, and one showed between 1% and 20% serotype A. The mean percentage A among the 13 cultures was 0.2% (calculated from the original data, rather than the frequency distribution shown in the figure). A control injection consisting of the *G* gene alone produced 0.7% rescue (Figure 1). The data show that pSA 1.56 (with insert from 5464 to 7026) and pSA 4.8 kb (7026–11825) both show 0.5% and are comparable to the control levels. Thus, we conclude that there is no evidence for rescue induced by fragments upstream of position 456 or downstream of 5464.

More than one region of the *A* gene can rescue: Reference to Figure 1 shows that pSA 5.4 (13–5464) was very effective in rescue (57.8%). The upstream half of this fragment, pSA 3.0 (13–2971), was also active, giving 7.4 and 39.5% in two different experiments. The nonoverlapping downstream half of pSA 5.4 (pSA 2.5, 2971–5464) was highly effective (55.9%). Similarly a number of other fragments ending at 4517 show good activity. Still another region downstream of these fragments (pSA 2.4, 4655–7026) showed a weak ability to rescue (3%), although clearly higher than control values.

Central repeat region is highly effective in rescue: pSA 1.0 (3517–4517) showed values of 42.2 and 43.8% in two experiments (Figure 1). The central region of the *A* gene contains about 3.5 copies (732 bases) of a 221 bp repeat extending from 3547 to 4278. Hence 73% of the 1.0 fragment consists of these repeats. Even a single copy (pSA 0.2) of the repeat gave significant rescue (6.8 and 13.9%). A single copy of the corresponding repeat from the 156*G* gene was injected in this same experiment as a control. It gave only the background value of 0.3%.

Longer regions are more effective than shorter regions: Figure 1 shows the results of injecting a series of plasmids with inserts terminating at base 4517. The longest of these is contained within pSA 4.5, beginning at 13. The others begin progressively farther downstream, the



NUMBER OF CLONES

PLASMID \ % A	0%	1% to 20%	21% to 40%	41% to 60%	61% to 80%	81% to 100%	MEAN %
pSA1.5	12	1	0	0	0	0	0.2%
pSA5.4	0	3	1	2	2	6	57.8%
pSA2.5	0	1	3	3	7	1	55.9%
pSA3.0	3	12	1	0	0	0	7.4%
	0	3	9	3	3	0	39.5%
pSA4.5	0	1	1	1	6	8	75.8%
pSA3.4	0	1	3	9	6	5	60.6%
pSA2.1	0	0	3	6	1	1	50.0%
	0	2	2	6	5	3	57.5%
pSA1.6	0	0	3	3	2	0	52.7%
	0	0	2	3	2	0	51.0%
pSA1.0	0	2	5	3	2	0	42.2%
	0	1	4	2	0	1	43.8%
pSA0.3	1	12	0	0	0	0	4.3%
pSAR1	0	11	0	0	0	0	6.8%
	0	16	3	1	0	0	13.9%
pSAR3.9	0	0	6	13	8	1	51.8%
pSA2.4	1	7	0	0	0	0	3.0%
pSA1.56	12	5	0	0	0	0	0.5%
pSA4.8	29	4	0	0	0	0	0.5%
pXΔ3	12	6	0	0	0	0	0.7%
pSGR1	20	5	0	0	0	0	0.3%

FIGURE 1.—Rescue of d48. A map of the *A* gene is given on the left of the figure along with the position and size of the test fragments. The heavy line represents the translated portions of the gene, the thin lines represent flanking regions and the open line is the region of the 221-bp internal repeats. The table at the right of the figure gives the number of clones having the indicated frequency of cells of serotype A. Cells showing serotype A are rescued. The mean percent of serotype A within the lines (a measure of efficiency of rescue) is given in the last column.

last contained within pSA 0.3. It is seen that the fragments become decreasingly effective in rescue, starting with a high of 75.9% for pSA 4.5 and ending with 4.3% for pSA 0.3.

DISCUSSION

Large segments of the *A* gene are more effective in rescue than smaller. Is this because they have more active regions or is it merely because of their size? Clearly there must be more than one active region, for nonoverlapping adjacent regions can each be effective by itself. Moreover, in some cases very small percentage changes in total size can have a large effect on rescue, which would not be expected if gross size is the determinant. It would be expected if some regions have higher activity than others. In the accompanying paper by YOU *et al.* (1993) it is shown that two adjacent nonoverlapping pieces of the *A* gene injected together are more effective than either injected alone. Thus effects can be cumulative, even when on separate pieces of DNA.

The possibility that different sizes of injected fragments may affect copy number in transformants has not been specifically ruled out. However, this possibility is unlikely, for previous work on transformation and copy number (KIM *et al.* 1992) has shown that within the

range of sizes considered here, copy number depends only on the number of fragments successfully introduced at the time of microinjection, copy numbers being maintained at a relatively constant value.

We conclude that the portion of the *A* gene effective in rescue is very large, with the most active section centered on the 221 bp repeats in the center of the gene, the larger the region, the greater its activity. There is no evidence for transcription of the fragments (JESSOP-MURRAY *et al.* 1991; YOU *et al.* 1993). Nevertheless, it has been shown by KOIZUMI and KOBAYASHI (1989) that a factor appears at autogamy in the cytoplasm of wild type cells (but not in the cytoplasm of vegetative cells) that will rescue d48 when injected into newly forming macronuclear anlagen. It is tempting to suggest that portions of the *A* gene DNA itself escape from the old disintegrating macronucleus and make their way through the cytoplasm to the newly developing anlagen where they base pair with *A* genes from the micronucleus and lend stability and direction to telomere formation and the removal of internal eliminated sequences recently discovered within the *A* gene (PREER *et al.* 1992).

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