

We have succeeded in inducing specific agglutination between cilia detached from the cells of complementary mating types.

Material and Methods

Cells of stock Ksy 1 and d-N14a which belong to mating types V and VI respectively in *Paramecium caudatum*, syngen 3, were used. Cilia were detached from mating-reactive cells by the treatment with Triton X-100 solution [7] and Ca as follows. About 8×10^6 mating-reactive cells were washed by slow centrifugation (500 rpm, 2 min) in Dryl's solution [8] containing 1 mM KCl and then treated with ice-cold Triton X-100 solution which contained 0.01% (v/v) Triton X-100 (octylphenoxypolyethoxyethanol), 20 mM KCl, 10 mM of the tripotassium salt of EDTA (ethylenediaminetetraacetic acid) and 10 mM tris-maleate buffer (tris (hydroxymethyl) aminomethane and maleic acid, pH adjusted to 7.0 by NaOH). After 30 min of this treatment, the cells, then killed, were centrifuged (500 rpm, 2 min) and transferred into ice-cold 10 mM tris-maleate buffer (pH 7.0) containing 20 mM CaCl_2 . In 1 or 2 min in this solution almost all cilia were detached from the cell bodies. The deciliated cell bodies were precipitated by slow centrifugation (500 rpm, 2 min). The supernatant was then filtered through a sheet of filter paper (Toyokagaku No. 2). The filtrate, an almost pure suspension of cilia, was centrifuged at 9 500 rpm (6 500 g) for 30 min. Precipitated cilia were washed by the same centrifugation in 10 mM tris-maleate buffer containing 10 mM CaCl_2 . The cilia of stock Ksy 1 (mating type V) thus obtained were strongly effective in inducing mating agglutination and conjugation in living cells of stock d-N14a (mating type VI), and vice versa.

When the cilia of both mating types thus obtained were mixed, they showed strong agglutination which was easily observable under the phase contrast microscope. Samples were fixed with 10% (v/v) formalin and, after chromium shadowing, were observed under the electronmicroscope (JEOL T-7) (fig. 1). Large agglutinations of cilia were observed in the mixture while no agglutination was observed in the unmixed control. When deciliated cell bodies from both mating types were mixed, or when those from one mating type were mixed with mating-reactive living cells of the complementary mating type, no agglutination was observed. The result clearly shows that mating agglutination in *Paramecium* occurs between ciliary surfaces of the cells of complementary mating types and the possibility

of participation of other regions of the cell surface in the reaction is excluded. Since the reaction system contains no living cells, the possibility of a soluble cofactor necessary for mating agglutination is also excluded.

We think that this is the simplest system of mating agglutination so far reported in protozoa and that it will be useful for the analysis of mating agglutination in eukaryotic microorganisms.

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Laser microdissection of actinomycin D segregated nucleoli

LINDA BRINKLEY¹ and M. W. BERNS², ¹Department of Oral Biology, School of Dentistry, University of Michigan, Ann Arbor, Mich. 48104, and ²Department of Developmental and Cell Biology, University of California, Irvine, Calif. 92664, USA

Summary. Nucleoli of tissue culture cells were segregated into their fibrillar (light) and granular (dark) components by treatment with actinomycin D. Following this segregation, the cells were treated with quinacrine hydrochloride, an agent which selectively sensitizes the nucleoli to argon laser light. The actinomycin D-segregated, quinacrine-sensitized nucleolar components (dark and light) were selectively irradiated with the laser microbeam and subsequent uridine uptake assayed. The data indicate that selective damage to the light (fibrillar) area is generally more damaging than damage to the dark (granular) area. These results support the idea that DNA is closely associated with the nucleolar fibrillar component.

The nucleolus is an organelle in which the relationship between structural organization

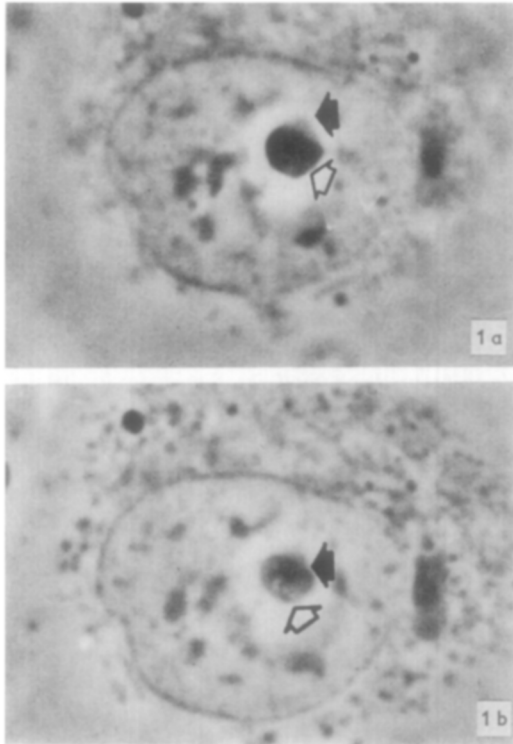


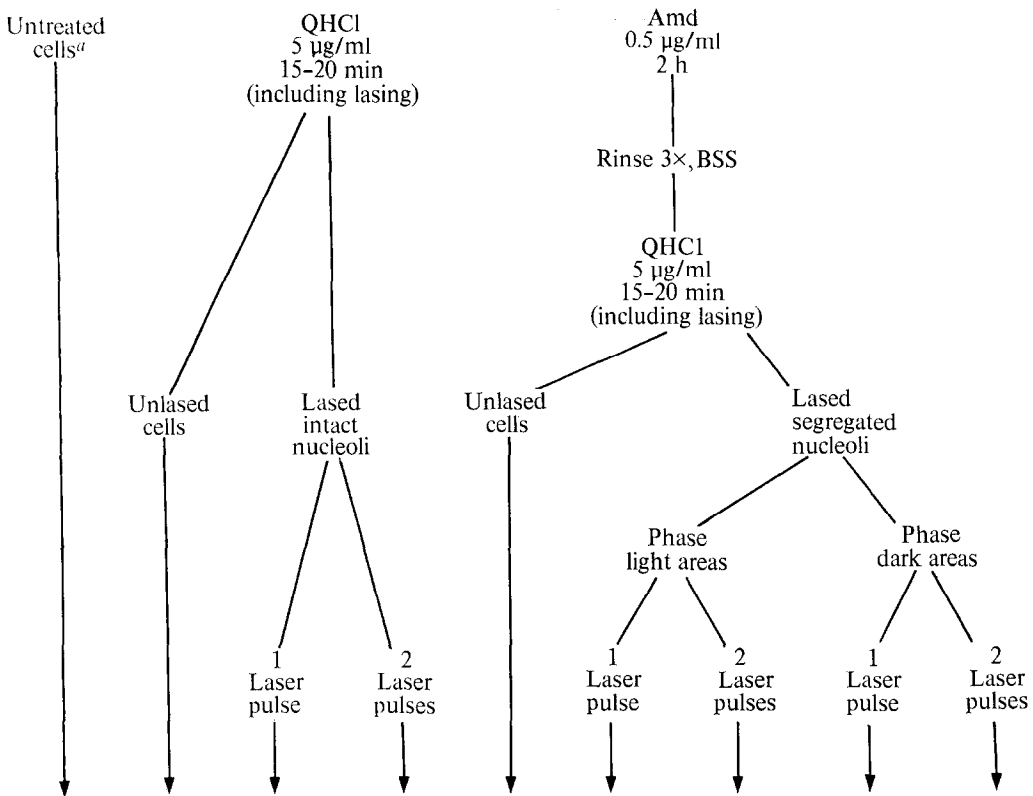
Fig. 1. Phase micrograph of live PTK₂ cells treated with actinomycin D (0.05 $\mu\text{g/ml}$) and quinacrine hydrochloride (5 $\mu\text{g/ml}$); (a) pre-laser, nucleolar segregation evident; open arrow, nucleolar dark area; solid arrow, nucleolar light area; (b) same cell post-laser irradiation of nucleolar dark area; note general damage and 'lightening' of this area; nucleolar light area appears unaffected and extends lightly out of the photographic plane.

and function still remains somewhat obscure. Whereas there is a vast amount of information deriving from studies on nucleolar ultrastructure and from studies employing various biochemical approaches, there is relatively little information derived from manipulated experimentation. One of the earliest and most useful experimental approaches used to elucidate nucleolar function was the study of Perry et al. [6] employing an ultraviolet microbeam. This was the first investigation that implicated the nucleolus in the formation of cytoplasmic RNA. In addition to the microbeam approach, an

often used method of studying nucleolar structure and function has been the application of the drug, actinomycin D. In low concentration (0.1 $\mu\text{g/ml}$), this drug inhibits nucleolar ribosomal RNA synthesis and causes a separation (segregation) of the fibrillar from the granular nucleolar components [3, 4]. The fibrillar component appears as phase light nucleolar material, and the granular component appears as phase dark nucleolar material. Both the ribosomal RNA and the segregation responses are reversible upon removal of the actinomycin.

The two above-mentioned approaches, microbeam irradiation of the organelle and actinomycin D treatment, have both proved useful. We have been combining both approaches. Instead of using the ultraviolet microbeam (which often proves generally deleterious to the cell), we have been employing an argon laser microbeam [2]. It has been demonstrated previously [1] that the nucleolus can be photosensitized to argon laser light by treatment with the antimalarial drug, quinacrine hydrochloride. Microbeam irradiation of nucleoli in quinacrine-treated cells results in structural and functional alteration of these organelles. The present study combines the laser microbeam-quinacrine technique with actinomycin D treatment. Nucleolar components were segregated by actinomycin D treatment, and then the various components were selectively irradiated.

In this communication, we will present data that (1) demonstrate that selective damage to intact nucleoli, nucleolar fibrillar, or nucleolar granular material results in different alterations in nuclear function and (2) demonstrate a dose response effect on function when the fibrillar and granular constituents are irradiated. The data support the idea that DNA is associated with the nucleolar fibrillar component.



1. Washed 3× with BSS; fresh medium added; allowed to recover 3½ h.
2. Incubated with 3H-UdK (50 µCi/ml; spec. act. 28.7 Ci/mM) – 30 min.
3. Rinsed 3× with BSS.
4. Fixed for AR.
5. Exposed 1 week.

^a This went from untreated cells to step 2.

Fig. 2. Experimental treatments: self-explanatory.

Material and Methods

Pt-K2 (*Potorous tridactylis*), cells which have a stable nucleolar number of one, were seeded into Rose chambers the day before an experiment. The medium consisted of Eagle's essential MEM, fortified with 10% fetal calf serum, and buffered with 0.85 g/l of NaH₂CO₃. The day of an experiment, the medium was changed to medium containing 0.05 µg/ml actinomycin D (Amd). When exposed to this concentration of Amd, nucleolar segregation occurred within 2–2½ h. Amd (Sigma, no. A-55-5, lot no. 1210-2000) was stored in aliquots at 0°C as a stock solution of 50 µg/ml. When segregation was evident, the Amd medium was withdrawn, the cells rinsed once with fresh medium, and medium containing 5 µg/ml QHCl was added. Nucleoli were laser-microirradiated after the quinacrine had been in the chamber 3–5 min. When the irradiation was completed, the quinacrine medium was removed and fresh medium

added. Total quinacrine exposure never exceeded 20 min. The argon laser microbeam system used for irradiation has been described elsewhere [2].

Fig. 1 illustrates segregated nucleoli before and after lasing. Following lasing, cells were rinsed 3 times in fresh medium and a fourth change of medium added. The cells were allowed to recover for 3–4 h, then 0.05 mCi/ml ³H-uridine (spec. act. 28.7 Ci/mM) was added. After 30 min, the cells were rinsed with BSS 3 times and fixed in Carnoy's for 15 min. Coverslips were cut out with a diamond pencil, run through an ethanol series to distilled water, incubated in 5% TCA at 4°C for 15 min, rinsed in distilled water, dehydrated through an ethanol series and air-dried. Coverslips were mounted in Permount, cell side up, on a glass slide. Slides were allowed to dry overnight, then dipped in Kodak NTB3 emulsion diluted 1:1 with distilled water, placed in a slide box with dessicant and exposed at 4°C for 7 days. Slides were developed in Kodak D-19 at 19°C for 2 min, rinsed briefly in

distilled water, fixed for 4 min, washed in running tap water for 30 min, and finally placed in distilled water prior to staining. Cells were stained in acid fuchsin-aniline blue (0.2 and 0.1 g, respectively, in 100 ml of 1 N HCl for 5 min) and dehydrated through 95% and absolute ethanol.

The following experiments and controls were done as outlined in fig. 2: (1) untreated labelling controls; (2) quinacrine only, lasered and unlased cells; (3) Amd followed by quinacrine, light or dark areas lasered, and unlased cells. Controls for lasered cells with a given treatment were the unlased cells in the same chamber. The nuclear labelling patterns of lasered and unlased control cells in the same chamber were evaluated by scoring the cell for whether the nucleolus, nucleus, or both, were labelled. Scoring was as follows: no nucleolar label (-NO), no nuclear label (-NU), nucleolar label (+NO), and nuclear label (+NU). Cells examined were assigned to one of three possible categories: (1) no nucleolar or nuclear label (-NO, -NU); (2) no nucleolar label, nuclear label present (-NO, +NU); and (3) both nucleolus and nucleus labelled (+NO, +NU). In the initial quantitation of the labelling patterns, cells were categorized by the number of autoradiography silver grains according to the following scheme: 0-1 grains, -; 2-10 grains, +; 11-30 grains, ++; more than 30 grains, +++. However, it soon became clear that cells were generally falling into the -, or ++, to +++ range. Consequently, in the presentation of data in this paper, cells are designated either (-) which is equivalent to the first category above, or (+) which is equivalent to any of the three categories (+), (++) or (+++).

Results

Table 1 contains the data for unlased control cells which were either untreated, exposed to quinacrine alone, or sequentially exposed to actinomycin D followed by quinacrine. The three treatments produce similar labelling patterns—about 88% of the cells recover and incorporate uridine in both nucleolus and nucleus following either drug treatment. Since control values were found to be similar

Table 1. Unlased controls

	% Cells with labelling pattern			(n)
	-NO -NU	+NO +NU	+NO +NU	
Untreated	1.5	12.3	86.2	(65)
QHCl	0.8	10.2	89.0	(118)
Amd-QHCl	1.9	15.9	89.2	(214)

$\chi^2 = 2.87$, d.f. = 4, $P = 0.58$.

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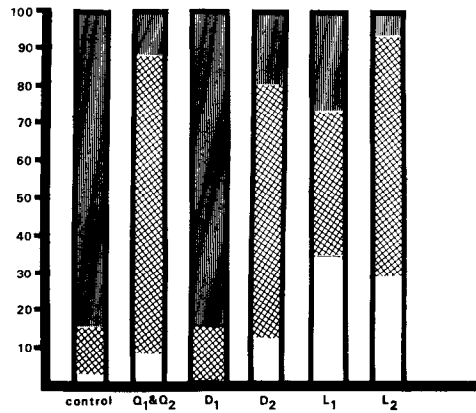


Fig. 3. Ordinate: % of cells with labelling pattern; □, no nucleolar or nuclear label; ▨, no nucleolar label, nuclear label present; ▤, both labelled.

Dose response: Q₁, quinacrine plus one laser shot; Q₂, quinacrine plus two laser shots; D₁, dark areas lasered, one laser shot, quinacrine and actinomycin D treatment; D₂, dark areas lasered, two laser shots, quinacrine and actinomycin D treatment; L₁, light area lasered, one laser shot, quinacrine and actinomycin D treatment; L₂, light area lasered, two laser shots, quinacrine and actinomycin D treatment.

($p = 0.58$), they were combined for statistical comparison with the labelling patterns of lasered cells.

Control patterns are compared to labelling patterns observed in lasered intact, lasered dark, or lasered light areas in table 2. The following observations are made: (1) lasering the intact nucleolus is most effective in impairing only nucleolar function, 84% of the cells do not have a nucleolar label but do have nuclear label; (2) lasering light areas is about 3 times

Table 2. Unlased controls vs Q, D, and L

	% Cells with labelling patterns			
	-NO -NU	+NO +NU	+NO +NU	(n)
Unlased controls	1.5	13.6	84.9	(397)
Q (Lased intact nucleolus)	6.2	84.4	9.4	(64)
D (Dark area)	10.9	63.6	25.5	(55)
A (Light area)	31	55.2	13.8	(58)

$\chi^2 = 299.7$, d.f. = 6, $p = 0.001$.

as effective as lasing dark areas and 5 times more effective than lasing intact nucleoli in blocking uridine uptake in both nucleolus and nucleus; (3) lasing dark areas has the least overall effect on both nucleolar and nuclear labelling; (4) when compared with the control value (84.9%), all of the nucleolar lasing experiments results in a significant inhibition of nucleolar and/or nuclear function.

Results broken down by dose-response are shown in fig. 3. Doses were one or two pulses (100 or 200 μ l) to the same spot. No dose-response phenomenon was seen when intact nucleoli were lased: one or two pulses to intact nucleoli (Q1 and Q2) cause essentially the same effects ($p=0.74$)—about 80% of such cells show blocked nucleolar incorporation of uridine. A significant dose-response is seen, however, when light or dark areas are lased. One pulse to a dark area (D1) produces a pattern similar to control values, whereas two pulses to a dark area (D2) result in a 63% increase in cells with no nucleolar label. Likewise, two pulses to a light area (L2) result in a 20% increase in cells with unlabelled nucleoli when compared with one pulse to the same area (L1). D2 is similar to L2 in the effect it produces in reducing nucleolar label alone ($-NO$, $+NU$) with approx. 65% of cells hit in either area showing this pattern. However, two pulses in light areas are about twice as damaging to the entire nucleolar-nuclear complex ($-NO$, $-NU$) as the same energy delivered to a dark area. When the overall effect on a cell is considered, i.e., the total damage or lack of it, a pattern emerged indicating that both the kind of lased area and the total energy are important. The least damage ($+NO$, $+NU$) is seen with one pulse to the dark area. A general progression of damage is seen as follows: $D_1 > L_1 > D_2 > L_2$. If results are examined for only nucleolar damage ($-NO$, $+NU$), $Q > L_2 >$

$D_2 > L_1 > D_1$. Lasing intact nucleoli results in the most specific nuclear damage.

Discussion

Cells are able to recover and incorporate uridine after treatment with quinacrine or actinomycin D followed by quinacrine. However, distinct disturbances of uridine uptake are seen as a result of lesions produced with the laser microbeam.

Lasing intact quinacrine-treated nucleoli results in the greatest selective nucleolar effect (i.e., nucleolar label reduced but nuclear label present) and also shows no difference in response between a dose of 100 or 200 μ J. This nucleolar selectivity is in sharp contrast with the effects of laser damage to light or dark nucleolar areas. About one-third of all cells lased in light areas show impaired RNA metabolism in both nucleolus and nucleus. This is a five-fold increase over damage seen in intact nucleoli and a three-fold increase over that seen when dark areas are lased. However, despite this significant effect on nucleolar and nuclear function, the highest percentages of effect were on the nucleolus alone.

The results indicate disparate effects when either area is lased. Laser damage to light areas is more generally damaging. This suggests that the light area may have DNA associated with it. This observation was suggested in earlier EM autoradiography experiments of Granboulan & Granboulan [5], which suggest that the pre-ribosomal particles are in the fibrillar area (equivalent to our light area) and move to the granular area prior to moving out the nucleolus. Since the large ribosomal unit would be formed in or near the DNA template, these observations would seem feasible.

Although the actual mechanisms of laser interaction with photosensitizing agents are not known, these results indicate that com-

bination of laser microirradiation and metabolically active drugs can be used to structurally microdissect and qualitatively examine nucleolar function. In general, our results support the idea that Amd-induced nucleolar segregation causes nucleolar DNA to be segregated from other components and probably into phase light area. Also, because of the high rate of both nucleolar and nuclear inactivation when lasing the light area of a segregated nucleolus, it is possible some extranucleolar DNA may be associated with or contiguous to these areas.

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Presence of two active X chromosomes in hybrids between normal human and SV40-transformed fibroblasts from patients with the Lesch-Nyhan syndrome

C. M. CROCE and B. BAKAY, *The Wistar Institute of Anatomy and Biology, Philadelphia, Pa 19104, and Department of Pediatrics, University of California at San Diego, La Jolla, Calif. 92037, USA*

Summary. Skin fibroblasts (LNSV) derived from a hypoxanthine-guanine phosphoribosyltransferase (HGPRT) deficient patient with the Lesch-Nyhan syndrome, who has glucose-6-phosphate dehydrogenase (G6PD) type A, were transformed with SV40 and hybridized with WI38 human diploid fibroblasts derived from a female embryo which have normal HGPRT and G6PD type B activities. The hybrid clones selected in hypoxanthine, aminopterin and

thymidine (HAT) medium, were essentially tetraploid and contained three X and one Y chromosomes. These hybrids contained HGPRT, types A and B and the AB heteropolymeric form of G6PD enzymes which were indicative that in these cells X linked genes of both parental cells were fully active. Hybrids back-selected in medium containing 8-azaguanine (8-AG) contained only two X chromosomes. They had no HGPRT activity and they contained only G6PD type A enzyme. It is concluded that the hybrid cells which grew in the presence of 8-AG retained the X chromosome of the LNSV parental cell and apparently the inactive X of the WI 38 cell.

Human diploid fibroblasts have a limited life span and can be carried in tissue culture only through a limited number of passages [1]. In contrast, hybrids of human diploid fibroblasts and human cells transformed with Simian virus 40 (SV40) behave as continuous cell lines and do not display the density-dependent inhibition of cell growth that is characteristic of normal human fibroblasts [2]. Fusion of normal human diploid fibroblasts containing normal HGPRT and G6PD type B activities, with SV40-transformed fibroblasts derived from a patient with the Lesch-Nyhan syndrome who lacks HGPRT activity, and who has G6PD type A, produced hybrids which were polyploid and expressed the X-linked genes of both parental cells.

Materials and Methods

Cells. WI38 cells are human diploid fibroblasts derived from a female embryo [3]. LNSV are human fibroblasts derived from a skin biopsy of a patient (III₃) with the Lesch-Nyhan syndrome [4] which have been transformed by SV40 [5].

Fusion. The cells were fused in the presence of β -propiolactone-inactivated Sendai virus in Eagle's minimal essential medium (MEM) at pH 8.0 as previously described [6].

Selection. After fusion, cells were maintained in HAT selective medium [7] to prohibit the growth of HGPRT-deficient cells and then subcultured to eliminate the slow-growing WI38 fibroblasts. Colonies of hybrid cells were picked 4-6 weeks after fusion. These cells were then cloned in microtest plates.

Back-selection. Several clones of cells growing in HAT medium were back-selected by growth in MEM containing 30 μ g/ml of 8-AG. Approximately one cell in 10⁶ gave rise to a colony in this medium.