
Plasmid migration using orthogonal-field-alternation gel electrophoresis

Robin C.Hightower, David W.Metge¹ and Daniel V.Santi*

Departments of Biochemistry and Biophysics, and Pharmaceutical Chemistry, University of California, San Francisco, CA 94143 and ¹Department of Microbiology, San Francisco State University, San Francisco, CA 94132, USA

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ABSTRACT

The migration properties of a series of supercoiled plasmids ranging in size from 4 to 16 kilobases (kb) have been analyzed by orthogonal-field-alternation gel electrophoresis (OFAGE). These circular DNAs enter the gel and are well resolved. Unlike linear DNA molecules, the relative mobilities of these plasmids are constant over a wide range of pulse times, from 10 to 120 seconds, as well as over a broad range of total running times, from 6 to 24 hours. Electrophoresis of supercoiled, relaxed, and nicked open circular forms as well as topoisomers of pBR322 shows that the extent of supercoiling has a dramatic effect on plasmid migration on OFAGE. Several practical applications for exploiting the different migration properties of circular and linear DNA molecules on OFAGE are presented.

INTRODUCTION

Pulsed field gel electrophoresis, a technique for the resolution of large linear DNA molecules, has developed rapidly over the last few years (1). Schwartz and Cantor (2) described "pulsed field gradient gel electrophoresis" (PFGE) which resolves DNA molecules from 20 to 2000 kilobases by applying pulsed, alternating, orthogonal electric fields to the gel. Subsequently "orthogonal-field-alternation gel electrophoresis" (OFAGE) (3), "field inversion gel electrophoresis" (FIGE) (4), and electrophoresis using a "contour-clamped homogeneous electric field" (CHEF) (5) were introduced which are variations of the PFGE method. On these gel systems, intact chromosomal DNA molecules have been fractionated from a number of lower eukaryotes including *Saccharomyces cerevisiae* (2,3,5) and several parasitic protozoa (6,7).

Migration and resolution of linear DNA molecules by these methods are functions of pulse time (the time interval for which the two alternate fields are applied), total running time, applied

field strength and the electric field geometry of the apparatus used (8,9). The migration of linear DNA molecules has been shown to be pulse time-dependent, therefore gels that are electrophoresed at a particular pulse time result in maximal separation of molecules in a specific size range (2,3,9).

The behavior of supercoiled plasmids has not been systematically investigated in these gel systems. However, Schwartz and Cantor (2) detected the 2 μ plasmid from *S. cerevisiae* using PFGE, and recently Garvey and Santi (10) showed that extrachromosomal circular DNA molecules from drug-resistant *Leishmania major* are resolved on OFAGE. These observations indicated that circular DNA molecules do migrate on these gel systems, and prompted us to investigate the properties of circular DNA molecules on OFAGE. In the present work, we have shown that supercoiled DNAs enter OFAGE gels and we have determined the relative mobilities of 4 to 16 kb supercoiled plasmids on OFAGE as a function of pulse time and total running time. In contrast to linear DNA molecules, the relative mobilities of these plasmids are constant over a broad range of pulse times and total running times. The absolute distance of plasmid migration is relatively independent of pulse time and generally proportional to total running time. With respect to plasmid conformation, three forms of pBR322 migrate in the following order on OFAGE: supercoiled >> relaxed > nicked open circular. Topoisomers are resolved between the supercoiled and relaxed forms of pBR322. Therefore, the extent of supercoiling has a dramatic effect on plasmid migration on OFAGE.

MATERIALS AND METHODS

Materials

The OFAGE units that were used have been described previously (3,10). Calf thymus topoisomerase I and the supercoiled plasmid ladder (containing 2.067, 2.972, 3.990, 5.012, 6.030, 7.048, 8.066, 10.102, 12.138, 14.174, and 16.210 kb plasmids) were purchased from Bethesda Research Laboratories (BRL), and the 5.012, 10.102 and 16.210 kb plasmids were gifts from A. Arthur at BRL. *S. cerevisiae* strain 1939 was a gift from L.R. Perez at the University of California, San Francisco, and *S. cerevisiae* strain SFSU1 was a gift from F. Bayliss at San Francisco State

University. [α - 32 P] dCTP and Hybond-N nylon membranes were purchased from Amersham Corporation, and nick translations were performed using a kit from BRL. Agarose gels were prepared from SeaKem agarose (FMC BioProducts).

OFAGE

Agarose blocks containing intact chromosomal DNA from *S. cerevisiae* were prepared essentially as described (2). The only modification was to increase the final incubation of blocks in NDS (10 mM Tris, 500 mM EDTA, 1% lauroyl sarcosine, pH 9.5, and 2 mg/ml proteinase K) to 48 hours, and to wash the blocks three times in 200 mM EDTA, pH 8, prior to storage at 4°C. OFAGE was performed essentially as described (3). Under standard conditions, samples were applied to 1.5% agarose gels and electrophoresis was carried out in 0.6xTBE (53 mM Tris, 53 mM boric acid, 1.2 mM EDTA, pH 8.4) at 12°C for 22 hours. The buffer was recirculated and changed at approximately the midpoint of the run for optimal resolution of the DNA samples.

OFAGE experiments were performed in which the total running time was kept constant at 22 hours, and the pulse time used was 10, 20, 30, 45, 90 or 120 seconds. A second set of experiments was completed in which the pulse time was kept constant at 120 seconds, and total running time was 6, 8, 12, 16, 18, 20, 22 or 24 hours. Gels that were electrophoresed for each condition included the plasmid ladder, the 5 kb and 10 kb plasmids as a reference for locating the corresponding bands in the ladder, and a cell block preparation of *S. cerevisiae*. At least two gels were run for each condition.

TOPOISOMERASE I TREATMENT OF pBR322

Reactions (20 μ l) were incubated for 30 minutes at 37°C in 5 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM KCl, 0.5 mM dithiothreitol, 0.1 mM Na₂EDTA, 30 μ g/ml bovine serum albumin and contained 0.5 μ g pBR322 DNA, and dilutions of topoisomerase I (1,0.5,0.1,0.01 unit) (11). Reactions were stopped by addition of EDTA to 20 mM; after one minute, sodium dodecyl sulfate was added to 0.2% and proteinase K was added to 100 μ g/ml; samples were incubated at 37°C for one hour. Untreated and treated samples of pBR322 were electrophoresed on OFAGE, as described in the preceding OFAGE section. After observing DNA samples by ethidium bromide

staining, gels were soaked in 0.25 M HCl (2x15 min); rinsed with distilled water; soaked in 0.5 M NaOH, 1.5 M NaCl (2x30 min); and 1 M Tris-HCl, pH 8.0, 1.5 M NaCl (2x30 min). DNA was transferred from the gel to Hybond-N (12), and the filter was hybridized with ^{32}P -labelled pC1 (13) which contains pBR322 sequences. The hybridization and washing conditions used were as described (14).

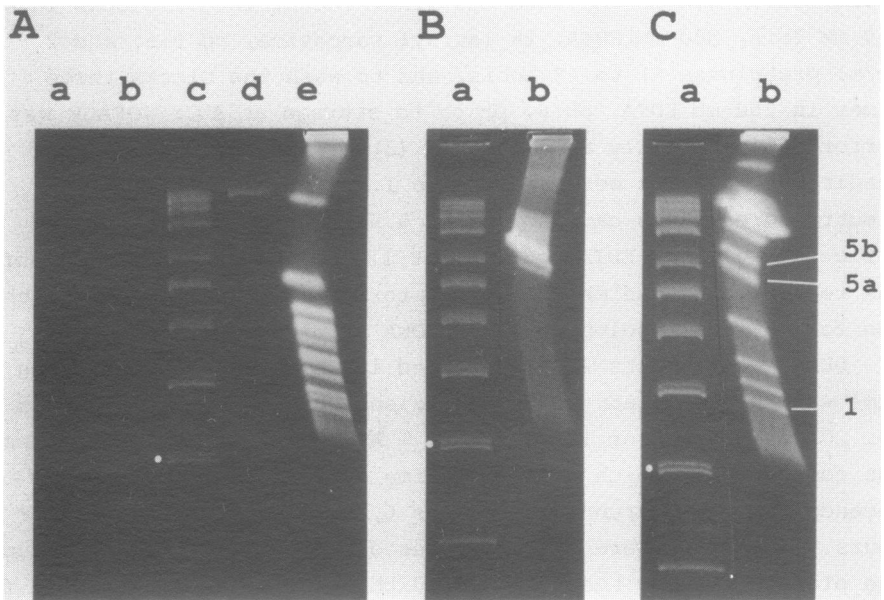


Figure 1. Comparison of the migration of supercoiled plasmids and yeast chromosomes on OFAGE. A. This gel was electrophoresed at a 120 second pulse time for 22 hours, and stained with ethidium bromide to visualize the DNA. The gel contains: (a) 0.1 μg supercoiled 5 kb plasmid; (b) 0.1 μg supercoiled 10 kb plasmid; (c) 0.3 μg supercoiled plasmid standards (BRL); (d) 0.1 μg supercoiled 16 kb plasmid; (e) cell block preparation of *S.cerevisiae*. The yeast chromosome banding pattern corresponds to published data (15). The order of plasmids in the ladder was determined by comparing the migration of the separate plasmids (lanes a,b,d) to the respective components in the ladder. B. OFAGE gel electrophoresed at a 10 second pulse time for 22 hours, and stained with ethidium bromide. These lanes from the gel contain: (a) 0.3 μg supercoiled plasmid standards; (b) *S. cerevisiae* cell block. C. OFAGE gel electrophoresed at a 45 second pulse time for 22 hours. Ethidium bromide stained gel lanes containing: (a) 0.3 μg plasmid ladder; (b) *S. cerevisiae* cell block. Chromosome bands 1, 5a and 5b are labelled. The 4 kb plasmid is indicated by a dot in A,B & C. The slower migrating band in (A), lane a, is the nicked open circular form of the 5 kb plasmid.

RESULTS**Migration of yeast chromosomes and the supercoiled plasmid ladder on OFAGE**

Fig. 1A shows an OFAGE separation of ten supercoiled plasmids ranging in size from 3 to 16 kb (lane c), and yeast chromosomes which range in size from 260 to 2200 kb (lane e) (15), that were electrophoresed under our standard conditions (120 second pulse time, 22 hours). The yeast chromosomes migrate approximately according to their size on OFAGE (16). By comparing the migration distance of separate 4.3 kb (pBR322, Fig. 3, lane a), 5, 10 and 16 kb plasmids (Fig. 1A lanes a,b, and d) to the respective components in the ladder (Fig. 3, lane f and Fig. 1A, lane c), it is apparent that supercoiled plasmid migration is inversely proportional to plasmid size on OFAGE. In this experiment, plasmids from approximately 4 to 16 kb and yeast chromosomes from 260 to 2200 kb resolve over the same region of the gel (roughly two-thirds the gel length), indicating that the size-mobility relationship of the supercoiled plasmids is quite different from that of the linear DNAs. With respect to linear DNAs of similar size to the supercoiled plasmids, 30 kb linear DNA migrates near the end of the gel under our standard conditions (10). At a 10 second pulse time for 22 hours, small linear DNA (3.3 to 12.8 kb) still migrates near the bottom of the gel, and much more rapidly than the supercoiled plasmid standards (data not shown). Therefore, in this study, the migration of plasmids in the ladder has been compared to yeast chromosomes that are resolved under the standard operating range of OFAGE.

Preliminary results suggested that the migration of these plasmids was not affected by variations in pulse times ranging from 45 to 120 seconds (17). The mobility of the supercoiled plasmids on OFAGE is described as a relative mobility value (R_f value) which is defined as the distance of the molecule's migration divided by the migration distance of the 4 kb plasmid in the ladder. We have observed that the migration of linear DNA molecules (2.0 to 23.1 kb), of similar size to the supercoiled plasmids used in this study, appears to be pulse time dependent but to a much lesser extent than large linear DNA molecules. For example, the absolute migrations of these linear DNAs at a 10

Table 1. Summary of Plasmid Migration on OFAGE.*

plasmid size	3.99	5.01	6.03	7.05	8.07	10.10	12.14	14.17	16.21
pulse time, sec									
10	1.00	0.75	0.57	0.47	0.38	0.31	0.28	0.26	0.24
20	1.00	0.77	0.58	0.46	0.37	0.27	0.23	0.20	0.19
30	1.00	0.76	0.58	0.46	0.38	0.29	0.25	0.22	0.20
45	1.00	0.74	0.57	0.46	0.37	0.29	0.25	0.21	0.19
90	1.00	0.75	0.56	0.44	0.35	0.25	0.22	0.19	0.16
120	1.00	0.74	0.56	0.44	0.35	0.25	0.21	0.19	0.17
mean	1.00	0.75	0.57	0.45	0.36	0.28	0.24	0.21	0.19
SE	0.000	0.004	0.004	0.005	0.005	0.010	0.010	0.010	0.011

*All values in the table represent relative mobilities (R_f) of the plasmids; R_f is defined as the distance of the molecule's migration divided by the migration distance of the 3.99 kb plasmid in the ladder. The R_f values for each running condition are the average of duplicate gels, except for 120 second pulse which represents the average of five gels.

Constant total running time, variable pulse time

second pulse time for 6 hours was 2.65 cm (2.0 kb) to 1.40 cm (23.1 kb), and at a 60 second pulse time for 6 hours was 3.00 cm (2.0 kb) to 1.55 cm (23.1 kb). However, OFAGE of small linear DNAs was not studied in detail due to the rapid migration of these molecules under our standard running conditions.

Effect of Pulse Time on Plasmid Migration

Electrophoretic separation of plasmids was examined as a function of pulse time. Table 1 summarizes the R_f values observed for plasmids resolved on OFAGE at a constant total running time (22 hours) and at several pulse times ranging from 10 to 120 seconds. These data show that the R_f values are quite constant for each plasmid at all pulse times examined. The largest plasmids (12,14,16 kb) migrate slightly slower and have slightly lower R_f values at higher pulse times. Overall, these data indicate that plasmid migration is independent of pulse time, in marked contrast to linear DNA mobility which is strongly influenced by alteration of pulse time (Fig. 1, Table 2).

The banding pattern of the plasmid ladder is reproducible at all of the examined pulse times, and the plasmids generally migrate to similar absolute distances on the gel when a constant running time is used (Fig. 1). For example, OFAGE of the 4 kb

Table 2. Summary of Yeast Chromosome Migration on OFAGE.*

yeast chromosome band	1	5a	5b
pulse time,sec			
10	0.42	NR	NR
30	0.75	NR	NR
45	0.81	0.40	0.35
120	0.84	0.69	0.67

*The values in this table represent relative mobilities (R_f) which were calculated as described in Table 1. The R_f values were determined at the specified pulse times for bands 1, 5a and 5b that correspond to *S. cerevisiae* chromosomes I, VIII, and V, respectively (15). The sizes of these chromosomes have been estimated to be 260 kb (1) and 580 kb (5a,5b) using OFAGE (16). Band 5a was determined to be slightly smaller than band 5b based on two other criteria: DNA content/meiotic bivalent and DNA content/chromosome (15). NR = not resolved. Refer to Figure 1 for the banding patterns of these chromosomes at three of the above pulse times.

plasmid for 22 hours and at pulse times ranging from 10 to 120 seconds showed an absolute migration of 5.24 ± 0.86 cm ($n = 15$).

Fig. 2 shows a plot of plasmid size vs. relative mobility for the 4 to 16 kb supercoiled plasmids that were electrophoresed on OFAGE over the range of pulse times examined (see Table 1). This plot illustrates the relationship between plasmid size and migration on OFAGE, and the curve which best fits these data is defined by the equation: $y = 6.144(x^{-1.316})$. The relative mobilities of the plasmids are constant over this broad range of pulse times, therefore the plot can be used as a standard curve for estimating the relative mobility of supercoiled plasmids between 4 and 16 kb on OFAGE under the specified electrophoresis conditions.

Effect of Total Running Time on Plasmid Migration

The effect of total running time on the separation of 4 to 16 kb plasmids was also examined. The results of plasmid migration in a series of OFAGE runs in which pulse time was kept constant at 120 seconds and the overall running time varied from 6 to 24 hours show that the R_f values of the plasmids are quite constant over this range of total running times ($SE \pm 0.003$ to ± 0.012). Further,

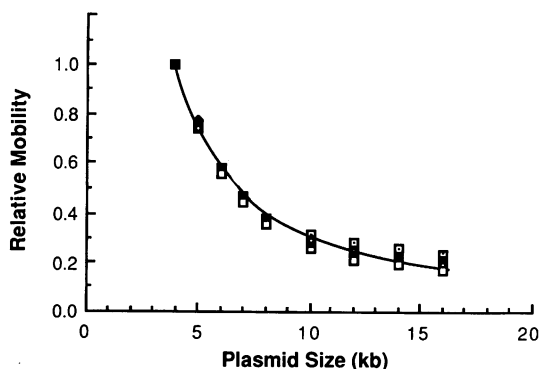


Figure 2. Plot of plasmid size vs. relative mobility for 4 to 16 kb plasmids. The curve was generated from the data set in Table 1 for gels electrophoresed at a constant total running time and a variable pulse time. The pulse times are represented by the following symbols: (■) 10 sec, (●) 20 sec, (■) 30 sec, (●) 45 sec, (■) 90 sec, and (■) 120 sec. The curve which fits these data is defined by the equation: $y = 6.144 \cdot x^{-1.316}$. This plot can be used as a standard curve for estimating the relative mobility of any supercoiled plasmid from 4 to 16 kb that is electrophoresed on OFAGE under the specified running conditions.

the mean relative mobilities of plasmids electrophoresed at variable pulse times and total running times are nearly identical (refer to Table 1).

Although the relative mobilities of the plasmids are constant at different total running times, the absolute migration increases with increased running time. Thus, the plasmid banding pattern is compact at short running times, and becomes more dispersed throughout the length of the gel as the total running time is increased (data not shown). Plasmids are optimally separated at run times of about 20 hours, and we use 22 hours as a standard time for our studies. In comparison to plasmid migration, the chromosomal DNA profile of *S. cerevisiae* is unresolved at run times less than 16 hours (data not shown). Approximately 11 bands are resolved across two-thirds the length of the gel at run times longer than 18 hours at a 120 second pulse time (Figure 1).

Effect of Superhelical Density on Plasmid Migration

The migration of supercoiled, nicked and relaxed forms of pBR322 were examined on OFAGE (Fig. 3). Relaxation of supercoiled pBR322 resulted in a dramatic decrease of mobility

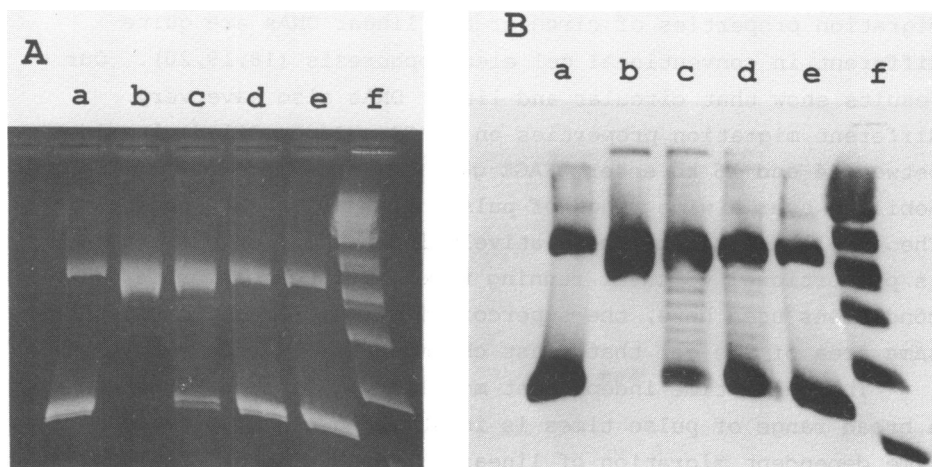


Figure 3. Migration of supercoiled, relaxed, and nicked open circular forms of pBR322 on OFAGE. A. Ethidium bromide stained gel with lanes a-e each containing 0.5 μg pBR322 DNA and the following concentrations of topoisomerase I: (a) no topoisomerase I; (b) 1 unit; (c) 0.5 unit; (d) 0.1 unit; (e) 0.01 unit. Lane f contains 0.3 μg supercoiled plasmid standards. B. Southern blot of gel in A to illustrate the ladder of topoisomers resolved on OFAGE (lanes c and d).

from $R_f=0.82$ to $R_f=0.43$ (Fig. 3, lanes a and b, respectively). The nicked open circular form of pBR322 migrated at a position ($R_f=0.40$) which was slightly slower than the relaxed form (Fig. 3, lanes a-e). Furthermore, topoisomers of pBR322 were resolved as a uniform ladder of bands between the supercoiled and relaxed forms on OFAGE (Figure 3B, lanes c and d). A comparison of supercoiled and relaxed forms of the 5 and 10 kb plasmids indicated that the relaxed forms of these plasmids also have a lower mobility than the supercoiled forms, but the migration of various forms of these plasmids has not been investigated in detail (data not shown). The above results show that the superhelical density of plasmids influences their migration on OFAGE, and that relaxed as well as nicked open circular forms migrate more slowly relative to supercoiled forms of the plasmid.

DISCUSSION

We have presented results that define the migration properties of plasmids on OFAGE. It is well established that the

migration properties of circular and linear DNAs are quite different in conventional gel electrophoresis (18,19,20). Our results show that circular and linear DNAs also have very different migration properties on OFAGE. Supercoiled plasmids between 4 and 16 kb enter OFAGE gels and show a constant relative mobility over a wide range of pulse times and total running times. The absolute mobility is relatively independent of pulse time but is proportional to total running time. Furthermore, under conditions used here, the supercoiled plasmids resolve over the same area of the gel that yeast chromosomes are resolved.

The pulse time independent mobility of plasmid migration over a broad range of pulse times is in striking contrast to the pulse time dependent migration of linear DNA molecules in the range of 20 to 2000 kb (2,3,8,21). Recently, pulse times have been shown to affect the optimal separation of linear DNAs extending down to 5 kb, implying that small linear DNAs are likewise sensitive to pulse time (22). We have also observed that the migration of 2 to 20 kb linear DNAs is influenced by pulse time, although the effect is small.

Based on the theoretical interpretation of DNA separation on PFGE, when molecules in the gel matrix are subjected to an electrical field that is perpendicular to their migration direction, the molecules are forced to reorient themselves along the new field and subsequently migrate forward. Smaller molecules will have a higher mobility than larger molecules because they are able to reorient more rapidly. Therefore smaller chromosomes are resolved at shorter pulse times than larger chromosomes which are resolved at longer pulse times (9). The plasmids we have examined are considerably smaller molecules than the smallest yeast chromosomes. We conclude that supercoiled plasmids between 4 and 16 kb appear to have similar abilities to reorient in the gel fields because their migration patterns are independent of pulse time.

The conformation of the plasmid also has a dramatic effect on its migration properties on OFAGE. Our results show that supercoiled, relaxed, and nicked open circular forms of pBR322 (4.3 kb) have strikingly different mobilities on OFAGE, with the following observed order of migration: supercoiled >> relaxed >

nicked open circle. The rapid migration of the supercoiled form has also been observed in conventional gel electrophoresis (18-20). Topoisomers of pBR322 are resolved on OFAGE as a ladder of bands between the supercoiled and relaxed forms which is the typical banding pattern for topoisomers on conventional gel electrophoresis (23).

There are several practical applications of this work. First, supercoiled plasmids between 4 and 16 kb can be used as migration reference standards on OFAGE, under the conditions described herein, because their relative mobilities are constant over a wide range of pulse times. Second, OFAGE with variable pulse times can be used for determining whether the mobility of a DNA is dependent or independent of pulse time, which can provide valuable information about its structure. For example, OFAGE provided the first indication that the amplified DNA in a stably resistant *L. major* cell line was extrachromosomal and circular (10). Finally, the pulse time-independent migration of these plasmids and the pulse time-dependent migration properties of linear DNAs may be exploited for optimal separation of circular DNA molecules from linear DNA molecules in a mixed sample (17). In principle, it should be possible to separate any circular DNA within the size of 4 to 16 kb from any linear DNA by variation of pulse time.

Our studies on the amplified DNAs in drug-resistant *L. major* have shown that supercoiled circular DNA molecules of around 30 and 60 kb are resolved on OFAGE and have mobilities similar to plasmids of around 7 to 8 kb (17). These observations suggest that large plasmids may have migration properties on OFAGE that are unusual and quite distinct from the smaller plasmids used here. We are currently extending these studies to analyze the OFAGE migration properties of plasmids larger than 16 kb to provide additional insight on the properties of nonlinear DNA molecules on OFAGE.

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*To whom correspondence should be addressed

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