At basal $[Ca^{2+}]_i$ the corrected values are 30 to 35 nM higher than what would be obtained with R_{min} and R_{max} from nonviscous calibration standards. The numerical effect of the above viscosity corrections happens to be quite similar to those used by Almers and Neher (*16*) but may vary with cell type and optical equipment.

14. Video images were acquired by a silicon-intensified target camera (model 66, Dage-MTI Inc., Michigan City, IN) mounted at the 35-mm camera port of an IM35 microscope that was equipped with a Nikon ×40 objective lens. The high voltage and gain of the camera were kept under manual control so that they were constant for any pair of wavelengths. The gamma correction in the camera was defeated so that camera output voltage would be directly proportional to light intensity. The TV camera output was fed both into a monitor screen and the image processor, an FD5000 from Gould Imaging and Graphics, San Jose, CA. All operations of the Gould FD5000 were controlled by a MicroPDP 11/73 (Digital Equipment Corp., Maynard, MA).

The FD5000 digitized the signal to 8-bit resolution and summed successive frames, typically 32, at each of the two excitation wavelengths. The summed wavelengths were normalized by the number of frames accumulated, and then prerecorded background images were subtracted to cancel out camera dark current and any autofluorescence due to microscope optics or in the media; these background images were acquired with the sample chamber filled with media but without cells. Cellular autofluorescence was not detectable at the excitation light levels and camera gain settings used. Once the two images of the cells at the different wavelengths were corrected for backgrounds, their sum was calculated in one memory bank while the difference of their logarithms was deposited in another memory bank. The difference of their logarithms or log-of-ratio between corresponding picture elements contains the information as to $[Ca^{2+}]_i$ level, but also reflects variations across the picture in the ratio between the strengths of the exciting beams. To compensate for these variations, a log-of-ratio image was prerecorded at the beginning of the experiment of a uniform thin layer of fura-2 solution in EGTA buffer trapped between two coverslips. Any nonuniformities in this log-of-ratio image of uniform dye indicated imbalances between the two excitation beams. Subtraction of this prestored log-of-ratio image largely corrected for these imbalances and normalized all ratios against the excitation ratio for the dye in EGTA buffer. Finally, the sum image and the corrected log-of-ratio image were merged into a single image containing 8 bits of information at each picture element—3 bits coded for the mean intensity of the two fluorescences and the remaining 5 bits coded for the corrected log ratio of the two fluorescences. The 3 bits of mean intensity controlled the brightness of the pseudocolor display over 8 possible values, while the 5 bits of log ratio controlled the hue over 32 shades from indigo through blue, green, yellow, orange, and red to magenta. Because the mean fluorescence intensity is coded as display brightness of each picture element, the image appears black between cells, even though the log ratio of the two signals, fluctuating near zero, is very noisy. Dim or bright regions of the output display show where the fluorescence was weak or strong, respectively, the latter giving more reliable $[Ca^{2+}]_i$ values. Acquisition of one complete pseudocolor image (512 horizontal × 486 vertical picture elements, each 8 bits) typically requires somewhat less than 3 seconds, of which 1.07 seconds is used for accumulating 32 frames at wavelength 1, 0.25 second for letting the image decay in the camera, 1.07 seconds for wavelength 2, and about 0.3 second to do the corrections and calculate the pseudocolor $[Ca^{2+}]_i$ image.

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Upstream Operators Enhance Repression of the *lac* Promoter

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To study regulation of transcription by distant elements, a wild-type *lac* operator was inserted upstream of a promoter-constitutive operator control region. The upstream operator is shown to aid in repression of transcription from the mutant control region. The effectiveness of the upstream operator as a function of its distance from the mutant control region parallels the length dependence observed for DNA cyclization. A quantitative model is proposed for action-at-a-distance of DNA control sites in which protein-protein and protein-DNA interactions are mediated by DNA looping. In this model, the effective concentrations of interacting proteins that are tethered by DNA are determined by the length of the intervening DNA and by its inherent bending and torsional stiffness. This model makes a number of predictions for both eukaryotic and prokaryotic control sequences located far from their sites of action.

THE *lac* OPERON HAS SERVED AS A paradigm for the molecular mechanism of gene regulation (*1*). Recently, however, eukaryotic systems have been described that appear not to conform to this classic model of direct interaction of activator and repressor proteins with RNA polymerase at the transcription start point (*2*). Specifically, regulatory sites have been identified that function irrespective of orientation and at large and variable distances from the site of initiation of RNA synthesis. In the *lac* operon, binding sites for *lac* repressor far removed from the classical operator site at the start of transcription have been identified (*3, 4*). Although in the wild-type operon these sites have had no proven role, we have proposed that they may aid in repression of a constitutive mutant (O^c) operator (*5*).

In this study we demonstrate that operators inserted at various distances upstream of a *lac* promoter–O^c operator pair enhance repression in vivo. Furthermore, this effect is length-dependent. The similarities between the distance dependence we observe for cooperative repression and that observed for DNA cyclization reactions (*6*) suggest a general mechanism for the regulatory action of distant DNA sites. Qualitative models involving DNA bending and looping have been proposed to account for the interaction of proteins bound to distant DNA sites (*2, 7–10*). For the arabinose operon of *Escherichia coli*, data obtained both in vivo and in vitro have been interpreted in terms of the looping model (*7*). Consideration of the three-dimensional distribution of DNA sites that are distant along the primary sequence of the DNA puts these models in a new light. Instead of merely impeding complex formation through the requirement for bending or distortion, the intervening DNA acts as a tether which can increase the concentration of one site (or its associated protein) in the vicinity of a second site, thus driving the interaction in a quantitatively predictable manner.

A *lac* promoter–constitutive operator control region has been fused to the galactokinase gene of pKO4 (*11*) to make the plasmid pKO^c (Fig. 1). The O^c mutation has thus been removed from the context of the *lac* operon and the pseudo operators that have been identified at the end of the *I* gene (*3*) and ~370 bp into the *Z* gene (*4*). To demonstrate the effect of distant operators directly, we constructed a series of plasmids with a wild-type *lac* operator placed at various positions upstream from the mutant operator in pKO^c. This configuration maximizes our chances of observing an effect, since an O^c operator in the control site both increases the basal level of expression and ensures that the auxiliary (O^+) site will be the preferred binding site for repressor. Table 1 lists the results of galactokinase and β-galactosidase activity measurements on cells

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containing these plasmids and a chromosomal copy of the *lac* operon in the presence or absence of the gratuitous inducer isopropyl-β-D-thiogalactoside (IPTG). In the absence of IPTG, placement of a wild-type operator upstream of the promoter–O^c operator results in a decrease in galactokinase activity (due to increased repression) and a concomitant increase in expression of β-galactosidase from the chromosome (resulting from the reduced level of free repressor in the cell due to binding of repressor by the additional operators on the plasmids).

Relative equilibrium constants for the plasmid operators can be obtained as follows. The ratio (P) of constitutive to induced (+1 mM IPTG) enzyme activity (E) is directly related to the equilibrium constant (K) for repressor (R)-operator (O) interaction (*12*). Assuming that expression is reduced by repressor binding simply in proportion to the equilibrium fractional occupancy of the primary (promoter proximal) operator site, then

$$P = \frac{E_{(-\text{IPTG})}}{E_{(+\text{IPTG})}} = \frac{[O]_{free}}{[O]_{total}} = \frac{[O]_{total} - [RO]}{[O]_{total}}$$

$$= 1 - \theta = \frac{1}{1 + K[R]} \cong \frac{1}{K[R]} \text{ for } P << 1$$

where $\theta = [RO]/[O]_{total}$, $K = [RO]/[R][O]_{free} = \theta/(1 - \theta)[R]$, [R] is the free repressor concentration, and K is an apparent equilibrium constant. Variations in K due to the presence of distant operator sites on the plasmid can be ascribed to a cooperative interaction between these sites. From concurrent measurements of P_{galK} and $P_{\beta gal}$ on the same cells (and thus with the same free repressor concentrations) we obtain relative binding constants from the relation $P_{\beta gal}/P_{galK} = K_{galK}/K_{\beta gal}$. Since in all cases the β-galactosidase is produced from the wild-type *lac* operon, comparisons of these ratios for different placements of the plasmid upstream operator provide valid relative binding constants for the O^c-repressor interaction in these constructs.

Upstream operators were found to increase the extent of repression of the plasmid promoter by as much as 40-fold for an O^+-O^c separation of 185 or 283 bp (Fig. 2). The striking observation, however, is that an O^+ located 118 bp upstream of O^c (center-to-center between operators) has only about a twofold effect on repression (*13*). This places severe restrictions on the model proposed to explain this phenomenon. Previous mechanisms for action-at-a-distance include cooperative linkage (by nonspecifically bound protein) between the upstream site and its site of action, cooperative conformational changes that are propagated along the DNA, and blockage of one-dimensional diffusion along the DNA. In each case one would expect a proximal binding site to be at least as effective as one located further upstream.

We propose that the increased repression of the *lac* promoter by the presence of upstream operator sites results from the cooperative effect of repressor bound to these upstream sites on binding of repressor to the O^c operator. Experiments that show that the *lac* repressor can interact with multiple DNA sites simultaneously have been reported (*14*). Thus, a cooperative interaction between distant operator sites can be obtained through the bridging effect of a *lac* repressor, made possible by the bending of the DNA into a loop structure. In this model the upstream site by itself is assumed to be ineffective in repressing transcription. It is only its effect on occupancy of the operator site overlapping the transcription start point that leads to increased repression. The fact that the upstream operator in pKO^+O^c 118 is relatively ineffective in aiding repression can be explained by the difficulty in bringing the two sites into the proper alignment due to the inherent stiffness of DNA over short distances.

Table 1. In vivo measurements of operator occupancy. Plasmid constructions are described in the legend to Fig. 2. HB101 (*hsd*S20 *rec*A *rps*L *lac*Y *leu pro gal*K)/F′*lac* ZU118 I^Q *pro*$^+$ cells containing the various plasmids were grown in MOPS medium (*23*) containing ampicillin (50 μg/ml), glycerol (0.2%), Casamino acids (0.2%), and thiamin (5 μg/ml). Overnight cultures were diluted 1/200 into duplicate flasks containing fresh media and either 0 or 1 mM DTT. After at least four generations in log phase growth, cells were chilled on ice, harvested by centrifugation, sonicated, and assayed for galactokinase activity, total cellular protein, and plasmid copy number by the methods of Adams and Hatfield (*24*). Plasmid copy numbers were calculated using the relations that 5×10^9 cells produce 1 mg of protein and that in minimal media cells should contain ~1.5 genomes per cell. Copy numbers in uninduced cultures were 100 ± 20 per genome and in induced cells 70 ± 20. β-Galactosidase assays were performed on the extracts according to Miller (*20*). The results of a representative in vivo operator occupancy experiment are listed. Variations in enzyme activities in duplicate experiments were within a factor of 2 of the reported values. However, ratios of galactokinase activity to β-galactosidase activity measured on the same cells were somewhat more reproducible, as reflected in the standard deviations listed in Fig. 2.

Plasmid	Galactokinase activity per (counts/min second per nanogram of plasmid DNA)		β-Galactosidase activity (OD_{420} per second per microgram of protein)	
	−IPTG	+IPTG	−IPTG ($\times 10^8$)	+IPTG ($\times 10^5$)
O^c	0.41	6.1	0.94	7.3
O^+O^c118	0.38	7.4	2.8	11
O^+O^c185	0.053	4.2	11	14
O^+O^c283	0.060	4.0	12	14

Data on the length dependence of cyclization of DNA fragments have been obtained by Shore and Baldwin (*6*). By measuring the kinetics of ligation of DNA fragments with complementary single-stranded ends, these authors have determined the cyclization probabilities (j) of linear DNA fragments of various lengths (Fig. 3). The parameter j represents the effective local concentration of one end of the molecule in the vicinity of the other; it exhibits a local maximum at ~500 bp. The theoretical curve of Shimada and Yamakawa (*15*) (calculated for a persistence length of 150 bp) (Fig. 3) indicates that j falls off rapidly as the chain length decreases below ~500 bp, but that j decreases only gradually with increasing chain length above ~500 bp. In fact, at 1361 and 4363 bp the observed values of j are ~50% and 20% of maximal, respectively. The physical explanation of this behavior is as follows. As the contour length is reduced toward the rigid rod limit [where the contour length (N) is less than the persistence length (L)], j drops steeply due to the inherent stiffness of the DNA. When N is $>10L$, however, the DNA molecule behaves like a flexible coil, for which j is approximately proportional to $N^{-3/2}$.

If one proposes that a *lac* repressor tetramer has the capacity to interact simultaneously with two operators on the same DNA molecule, then the situation should be directly analogous to the cyclization experiments discussed above. Alternatively, a *lac* repressor tetramer bound at each of the operators that can interact cooperatively with its counterpart would generate a similar length dependence. Although we know of no precedent for *lac* repressor association beyond tetramers, our observation of an increased level of β-galactosidase synthesis (indicating reduced free repressor levels) in cells containing pKO^+O^c185 and pKO^+O^c283 over those containing pKO^+O^c118 (Table 1) is consistent with a correlation between increased repression of galactokinase and increased repressor binding. Since the total number of repressor molecules and the plasmid copy number are approximately the same (within a factor of 2 to 4) and both substantially exceed the number of free repressor molecules in the cell (see below), small changes in the level of association to the plasmid operators could have large effects on the free repressor concentration.

For either model of the cooperative interaction, the effect on O^c occupancy of placing a strong repressor binding site upstream may be viewed as arising from an increase in

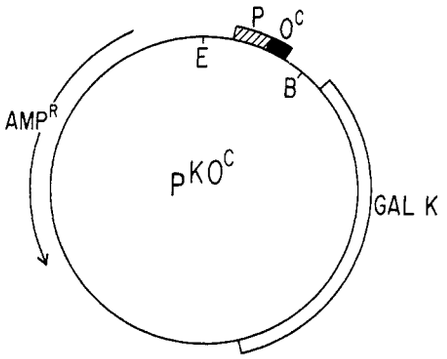

Fig. 1. Structure of pKO^c. A DNA fragment containing the *lac* promoter (hatched box) and operator (filled box) was inserted into the promoter-cloning vector pKO4 (*11*). The 283-bp Eco RI fragment from M13ΔE30^{c}666 (*5*) was digested with Hae III to give a 140-bp fragment and ligated between the Eco RI (E) site and the Bam HI (B) site of pKO4 (which had been filled in with the Klenow fragment of DNA polymerase) to make pKO^c.

the local concentration of *lac* repressor. In addition to the bulk value, the effective repressor concentration will include the concentration of repressor localized at the upstream site, specified by the *j* factor. The increase in O^c occupancy can then be attributed to the increased effective repressor concentration rather than to changes in the intrinsic binding constant, as discussed above. (Since $P \cong 1/K[R]$, an increase in K or in [R] or both will result in a decreased P value.) Note that this is an equilibrium model that postulates a bidentate ligand and a stable bridged complex. Sliding or other transfer mechanisms, which require dissociation of the protein from the distant site for stable complex formation at the control site, will facilitate both association and dissociation and hence will have no net thermodynamic consequence for occupancy.

If one repressor bridges between two operators, and if the binding sites on free and singly bound repressors have similar intrinsic affinities for operator DNA, the effective repressor concentration is inversely proportional to the galactokinase P values. The corresponding free repressor concentrations can be obtained similarly from ratios of β-galactosidase P values. The difference between the effective and free repressor concentrations can then be attributed to the *j* factor characteristic of the intervening DNA, provided occupancy of the upstream site is sufficiently close to 1. Free repressor concentrations in an i^+ strain of *E. coli* have been estimated to be ~1 n*M* (*16*). In our experiments in an i^Q background the free concentration may be as much as 10 n*M*. If we assume a free repressor concentration of ~5 n*M* for pKO^c-bearing cells, we obtain the results listed in Table 2. As seen for O$^+$O^{c}185 and O$^+$O^{c}283, if the product of the *j* factor and the occupancy of the upstream site is in sufficient excess over the free repressor concentration, the major contribution to occupancy of the O^c site will come from repressors already bound to the upstream site. The repressor concentrations arising from looping ($[R]_j$), plotted as a function of distance between operators (Fig. 3), are in reasonable agreement with the *j* values of Shore and Baldwin. Values of *j* observed in vitro may in fact be a significant underestimate of *j* inside cells, since the higher ion concentrations in *E. coli*, supercoiling, and excluded volume effects should all favor DNA bending and compaction. The finite size of the bridging repressor (or repressors) will also have an effect.

Whether we assume one or two repressor tetramers per complex and whether we attribute the effect to changes in the binding constant or the effective repressor concentration, the basic principles of the looping model remain the same. By tethering two sites that have the capacity to interact, one increases their relative concentrations and favors complex formation. The fact that DNA is a stiff tether introduces a local maximum in the relative concentrations as length is increased. For simplicity of the mathematical treatment we have assumed a stoichiometry of one repressor per O^c operator. As noted above, from the few measurements we have made thus far, there are already indications that the stoichiometry may be higher. Repressor titrations in vitro under tight binding conditions will be necessary to resolve this point.

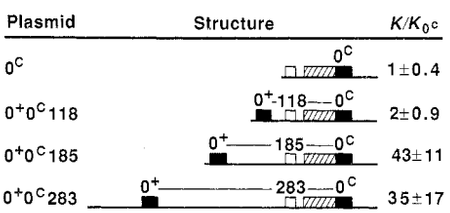

Fig. 2. Operator-containing plasmids and their relative equilibrium binding constants for *lac* repressor. Plasmids were constructed by standard techniques (*19*). The details of the constructions are listed below. *lac* (operator titration) and *gal* phenotypes of transformants were monitored on LB-XGal and MacConkey galactose plates, respectively (*20*). Structures were verified by restriction mapping and DNA sequencing (*21*) with a pBR322–Eco RI primer (Pharmacia). pKO$^+$O^{c}118 contains a 40-bp *lac* operator fragment (gift of Susannie Cheung) inserted at the Eco RI site of pKO^c. pKO$^+$O^{c}185 contains a ~67-bp fragment of unknown origin between a 40-bp *lac* operator and the Eco RI–Bam HI O^c 140-bp fragment. pKO$^+$O^{c}283 contains a 283-bp *lac* fragment bearing promoter mutation T743, constructed by recombination between λp*lac*5-T743 (*22*) and M13ΔE30$^+$ (*5*), inserted in the indicated orientation into the Eco RI site of pKO^c. CAP (open boxes), promoter (hatched boxes), and operator sites (filled boxes) are indicated. The center-to-center distance between operators is listed above the relevant constructions. Ratios of equilibrium constants (normalized to that of pKO^c) were determined as described in the text. Standard deviations of multiple (3 to 5) determinations are listed.

Table 2. Contributions to the effective repressor concentration. Repressor concentrations (nanomolar) were calculated from ratios of the P values obtained for O$^+$-containing plasmids to that obtained in the reference strain bearing pKO^c. The free repressor concentration, which is equal to the effective repressor concentration for pKO^c, is assumed to be 5 n*M* for cells containing the pKO^c plasmid. P values used in these calculations were averages of three to five determinations (the same data set as used in Fig. 2). Errors in $[R]_{effective}$ (determined from galactokinase P values) and $[R]_{free}$ (determined from β-galactosidase P values) are within ±50%. $[R]_j$ is calculated as $[R]_{effective} - [R]_{free}$.

Plasmid	$[R]_{Effective}$	$[R]_{Free}$	$[R]_j$
O^c	5	5	0
O$^+$O^{c}118	8	4	4
O$^+$O^{c}185	27	1	26
O$^+$O^{c}283	18	1	17

Johnson and Simon (*17*) have recently identified an "enhancer" site for the *hin* site-specific recombination system, which seems to act independently of position and orientation. A minimal spacing between the enhancer sequence and the site of recombination has been shown to be necessary for activity. This is directly analogous to our observation in *lac*. DNA flexibility constraints may limit protein-protein interactions at short intersite distances.

Promoters in the arabinose (*7*) and galactose (*8*) operons have been shown to be under the control of multiple operator sites. Further, experiments in which the spacing between the operator sites is altered in these systems have shown a periodicity of ~10 bp in the level of repression. The looping model predicts this behavior as a manifestation of a requirement for torsional alignment of the DNA sites. The data of Shore and Baldwin (*6*) show a strong periodic oscillation in *j* from 237 to 252 bp. In this range, the DNA is torsionally stiff enough so that achieving the proper alignment of the two ends in molecules with a nonintegral number of helical repeats requires a significant expenditure of free energy. For simplicity, the angle independent *j* factor ($j_{(0)}$), calculated from equation 50 of Shimada and Yamakawa (*15*), is plotted in Fig. 3. This curve does not reflect constraints due to torsional alignment and thus gives a maximal estimate of *j* in this range. More refined calculations by these authors include torsional constraints and show periodic oscillations that damp out at longer chain lengths as the distribution of orientations of the ends be-

comes broader. If a common mechanism underlies the multiple operator interactions in all three operons, we predict that a fine scale deletion analysis of *lac* should yield a ~10 bp periodicity, and that *ara* and *gal* should show a minimum interoperator distance for effective repression.

Hochschild and Ptashne (*9*) have reported a similar periodic oscillation in the extent of operator binding by λ repressor in vitro. In these experiments the spacing between sites ranges from 52 to 67 bp, over which distance the DNA is too stiff to make a complete loop without significant free energy costs (Fig. 3). Further, the authors see very little difference between cooperative effects when the interoperator spacing is reduced from 63 to 52 bp (a difference of ~1 helical turn). This result is difficult to reconcile with the precipitous drop in j with decreasing length (Fig. 3). A possible explanation of their results is that flexibility in the cI protein rather than in the DNA is the important factor in the interaction over these short distances.

Although the key observation in support of the DNA loop model is the diminished effect at short distances, flexibility on the part of the protein components of the system in principle can mask this lower limit. An observation that may be important in this context is the recent identification of a long COOH-terminal domain of a tandemly repeated seven–amino acid sequence on the largest subunit of eukaryotic RNA polymerase II (*18*). If this sequence adopts an extended structure as suggested (*18*), it may give eukaryotic transcription complexes the necessary flexibility to utilize upstream DNA control sites at short range.

The fact that points on a DNA chain several hundred to several thousand base pairs distant exist at high relative concentration to each other may prove to be important in understanding a number of protein-DNA interaction systems. Where binding sites quite distant from the site of action (such as the site of transcription initiation or site-specific recombination) can be moved or inverted with little effect on their control properties, the salient feature of the control system may indeed be the relatively constant high effective concentrations of proteins bound to those sites. This type of regulation at a distance provides a very flexible framework for control. Positive and negative regulatory proteins bound to their distinct sites may then compete for binding to a transcription complex (for example) according to the product of their relative concentration and affinity for the complex. At the DNA level, concentrations of accessory proteins might be increased either through increased affinity for upstream sites or through reiteration of binding sites. Relative concentrations might, in turn, be regulated temporally or developmentally by constraints on the flexibility of the intervening DNA. Supercoiling might increase the tendency for loop formation while rigid chromatin structure might decrease it. The *lac* system provides an entry point for testing these ideas in vivo and in vitro.

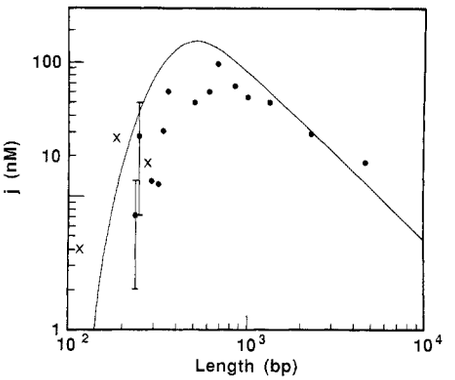

Fig. 3. Effective concentrations (j) as a function of the intervening length (bp). Filled circles (●) represent data of Shore and Baldwin (*6*) obtained from cyclization experiments on DNA restriction fragments. Periodic oscillations in j with length between 230 and 260 bp are represented by single points at 240 bp (average of six points 237–245) and 250 bp (average of six points 247–254) and error bars to indicate the range of the oscillations. The solid line is the theoretical curve for the local concentration of one DNA site in the vicinity of the second, calculated from equation 50 of Shimada and Yamakawa (*15*) (in the range of 10^2 to 10^3 bp) and from the Gaussian limit [$j \propto (\text{bp})^{-3/2}$ in the range from 10^3 to 10^4 bp] with a persistence length of 510 Å (150 bp). Local repressor concentrations at the O^c operator site, arising from a bound upstream repressor ($[R]_j$), calculated as described in the text, are plotted (X) as a function of center-to-center interoperator spacing.

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