Quality and position of the three lac operators of *E.coli* define efficiency of repression

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Repression of the lac promoter may be achieved in two different ways: either by interference with the action of RNA polymerase or by interference with CAP activation. We investigated cooperative repression of the *Escherichia coli* lac operon by systematic conversion of its three natural operators (O1, O2 and O3) on the chromosome. We find that cooperative repression by tetrameric Lac repressor increases with both quality and proximity of the interacting operators. A short distance of 92 bp allows effective repression by two very weak operators (O3, O3). The cooperativity of lac operators is discussed in terms of a local increase of repression concentration. This increase in concentration depends on flexible DNA which allows loop formation.

Key words: cooperativity/DNA loops/lac operator/Lac repressor/local concentration

Introduction

Cooperation between the two distant DNA sites is frequently observed in transcriptional regulation in pro- and eukaryotic organisms. It occurs in both negative and positive regulation. For *Escherichia coli*, it has been shown that at least two operators are necessary for full repression of the *araBAD* operon (Dunn et al., 1984), the *deo* operon (Dandanell et al., 1987), the *gal* operon (Haber and Adhya, 1988) and the *lac* operon (Oehler et al., 1990). In the *ginAlg* operon, activation depends on at least two NR binding sites (Reitzer and Magasanik, 1986).

The effect of a repressor on expression from a particular promoter may be increased by cooperative interaction. This implies that deletion or inactivation of a distant operator may have a negative effect on the action of a promoter-proximal operator. A simple quantitative explanation for such an observation is that a promoter-distal operator increases the occupation of the proximal operator by DNA looping (Mossing and Record, 1986; Schleif, 1988, 1992; Adhya, 1989, Law et al., 1993). The regulatory protein, which possesses two independent DNA binding sites, is then trapped in a ‘chelate-like’ complex (Hammer and Dandanell, 1989) between the two operators while the intervening DNA loops out. DNA loops have been shown to form *in vivo* for all of the above mentioned regulatory systems (Krämer et al., 1987, 1988; Ninfa et al., 1987; Amouyal et al., 1989; Lobell and Schleif, 1990), except for the *gal* operon—Gal repressor interaction (Brenowitz et al., 1990).

We have previously shown that the auxiliary operators O2 (Reznikoff et al., 1974), which lies 401 bp downstream of O1, and O3 (Gilbert et al., 1976), which lies 92 bp upstream of O1, contribute significantly to the repression of the *lac* operon, by cooperation with O1 (Eismann et al., 1987; Oehler et al., 1990; see Figure 1). Simultaneous binding of one Lac repressor tetramer to two *lac* operators (Borowiec et al., 1987) is necessary for full repression of ~1000-fold (Oehler et al., 1990). A DNA loop with either O1 and O2 or O1 and O3 is formed. Interestingly, the cooperative effect of each of the two auxiliary operators is about equally high, despite their different quality: O2 is weaker than O1 (Pfahl et al., 1979; Winter and von Hippel, 1981; this work) and O3 is weaker than O2 (Pfahl et al., 1979; Fried and Crothers, 1981; Winter and von Hippel, 1981; this work). The repression exerted by a particular operator depends on its affinity for Lac repressor and on the concentration of Lac repressor.

It has been shown that Lac repressor binds to the *lac* promoter region simultaneously with RNA polymerase (Schmitz and Galas, 1979; Straney and Crothers, 1987). Thus, the occupancy of an operator by Lac repressor at the position of O1 is directly correlated with its affinity for Lac repressor. For the influence of competition on binding by RNA polymerase see Lanzer and Bujard (1988). Repression of expression is defined as constitutive expression divided by expression under negative control of repressor. In a simple model, assuming that expression is proportional to the fraction of operator molecules free from repressor, the repression value F is related to the occupancy ([RO]/[O]) of an operator in the following way: F = 1 + ([RO]/[O]) (Sadler and Novick, 1965). Thus, repression values of 10 or greater can in practice be seen as directly correlated with the occupancy of an operator.

Here we present a detailed analysis of the influence of the qualities and distances of the *lac* operators on cooperative repression which, in our interpretation, depends on DNA loop formation.

Results

We constructed plasmids which put the *lacZ* gene under control of the natural *lac* promoter and the three *lac* operators (O1, O2 and O3). They were either unchanged or altered by site-directed mutagenesis. Each construct was cloned in plasmid λIP1 (Stieg et al., 1989). Single lysogens of an *E.coli* strain carrying deletions of the *lacZ*
and lacI genes (BMH 8117 F') were then generated with these phages. Specific β-galactosidase activities were determined in bacteria grown in the absence and in the presence of different amounts (five times or 90 times i^+') of plasmid-encoded Lac repressor.

**Comparison of the relative in vivo affinities of lac operators for Lac repressor**

In order to evaluate the relationship between operator quality and its contribution to DNA loop formation in vivo, the relative quality of the operators had to be determined in vivo. To that purpose, we measured repression of β-galactosidase expression by Lac repressor in the presence of just O3, O2 or O1 at the position of O1 (O2 and O3 have been destroyed at their natural positions; see legend to Figure 2).

The natural auxiliary operators (O2 and O3) were inactivated by site-directed mutagenesis to make any cooperative interaction impossible. The first lac operator O1 was replaced by O2 or O3 or the fully symmetric 'ideal' lac operator (Sadler et al., 1983; Simons et al., 1984; Lehning et al., 1987). See Figure 2 for the operator sequences. Figure 3 gives the repression values when about five times or ~90 times the i^+ amounts of dimeric or wild-type tetrameric repressor respectively are present. Dimeric Lac repressor is not encoded by the i^+ allele here, which we have described previously (Lehning et al., 1988; Oehler et al., 1990). Preliminary experiments indicate that i^+ encoded dimeric Lac repressor dissociates into monomers at low concentrations (data not shown). We therefore constructed another more stable (data not shown) Lac repressor variant (Lac R 331 Stop), by the introduction of a stop codon at position 331 of the coding sequence of the lacI gene. This removes the 30 C-terminal residues of Lac repressor which encompass both leucine heptad repeats (Oehler et al., 1990; Alberti et al., 1991). The heptad repeats of wild-type Lac repressor form a four-helical bundle and thus enable wild-type Lac repressor to aggregate to tetramers (Alberti et al., 1993).

The repression values of Figure 3 show that the affinity of O2 for Lac repressor is on average ~10-fold lower and...
that of O3 is ~300-fold lower than that of O1. In contrast, Oid binds repressor ~6-fold better than wild-type O1. These results are in general agreement with in vitro measurements. The in vitro affinity of O2 has been determined to be 1/10 of that of O1 (Pfall et al., 1979; Winter and von Hippel, 1981). The in vitro affinity of O3 has been determined to be between 16- and 1000-fold lower than that of O1 (Pfall et al., 1979; Fried and Crothers, 1981; Winter and von Hippel, 1981). The in vitro affinity of the ideal operator was found to be ~8-fold greater than that of O1 (Sadler et al., 1983; Simons et al., 1984).

**The role of O2 in repression**

We have shown that the natural O2 alone, in the absence of O1 and O3, works very inefficiently, if at all, as a roadblock for RNA polymerase (Oehler et al., 1990). Therefore, the effect which O2 exerts in the presence of a second operator at the position of O1 is predominantly due to cooperative interaction, i.e., DNA loop formation. An observed increase in repression by the addition of O2 should result from cooperative increase of occupation of O1 or any other operator at the position of O1. Figure 4 gives the repression values of O1, O2 and O3 at the natural location of O1 in the presence of O2. For comparison, the values for the constructs without the natural O2 from Figure 3 are also listed.

Dimeric Lac repressor is not able to form DNA loops (Oehler et al., 1990). Consequently, it shows virtually the same repression with and without the second operator, at both low and high Lac repressor concentrations. In contrast, repression by wild-type tetrameric Lac repressor...
Repression efficiency at three lac operators

Repression by Lac repressor

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Fig. 5. Repression factors for chromosomal (see Materials and Methods) lacZ genes. Repression values are given as the specific β-galactosidase activity in the absence of active Lac repressor divided by the specific activity in the presence of the indicated Lac repressors. For details see legend to Figure 3. A, λEwt 123; B, λEwt 103; C, λEwt 301; D, λEwt 003, this construct carries the operator mutant O1’ a; E, λEwt 001, this construct carries the operator mutant O1’ b which has been inserted to exclude any residual operator activity; F, λEwt 303; G, λEwt 101.

increases at low concentration with the addition of the second operator. With high concentrations of repressor, little effect of O2 can be seen. This is in agreement with the fact that at high repressor concentrations, DNA loops break up and cooperativity is lost (Krämer et al., 1987).

The cooperative effect of the second operator on O1 or O2 at the position of O1 is quite similar, namely 12- and 18-fold. The increase of repression by O3 at the position of O1 through cooperation with O2 cannot be directly determined, since repression of construct λEwt 300 (Figure 4, line E) by five times i⁺ amounts of repressor is too low to be accurately measured. Ninety times i⁺ amounts give a 16-fold repression with O3 at the position of O1 (λEwt 300, Figure 4, line E). Since the occupancy [RO]/[O] of an operator will increase linearly with an increase of Lac repressor, we can estimate that repression with five-fold i⁺ amounts should be 1.8. Then, the cooperativity of the second operator with O3 is ~7-fold, which is not far from the effect with O1 or O2.

The role of O3 in repression

When O2 at its natural position is inactivated, repression by low amounts of tetrameric repressor does not drop, because of the interaction between O1 and O3 (λEwt 123, Figure 5, line A and λEwt 103, Figure 5, line B). Naively, one might expect that, since cooperation is mutual, O1 and O3 could be exchanged without any effect on repression. But Figure 5, line C (λEwt 301) shows that repression drops ~6-fold, although it still remains relatively high compared with repression with dimeric Lac repressor. Repression of λEwt 001 (Figure 5, line E) reveals that part of the repression of λEwt 301 (Figure 5, line C) is obviously due to interference with CAP activation by Lac repressor bound at the position of O3.

This construct (λEwt 001, Figure 5, line E) indeed illuminates the difference between true repression and deactivation. While repression increases linearly with increasing amounts of Lac repressor, deactivation can maximally abolish the effect of an activator. In this case, deactivation by high amounts of Lac repressor is ~30-fold, which is close to the 50-fold reported value of CAP activation of lac promoter (Hopkins, 1974). Interestingly, dimeric repressor deactivates less well than wild-type tetrameric Lac repressor at both low and high concentrations. This could be explained by reduced steric hindrance of CAP binding by the smaller repressor protein. We have previously shown that high amounts of Lac repressor exert repression even with the natural O3 in the absence of both O1 and O2 (Oehler et al., 1990). This apparent repression is in fact deactivation by competition with binding of CAP to its target site immediately downstream of O3. In the absence of the CAP protein there is no repression of construct Ewt 001 by Lac repressor (Sundarp, 1993). If we take an ~20-fold deactivation into account, an ~40-fold true repression of construct λEwt 301 (Figure 5, line C) remains. Obviously, in a DNA loop the operator which blocks polymerase, rather than the overall strength of the cooperative interaction, determines repression. This reflects the fact that the loop is asymmetrically breathing. When O1 is now restored to its natural position (λEwt 101, Figure 5, line G), repression is increased with respect to the natural situation (λEwt 103, Figure 5, line B). Repression by dimeric repressor, where there should be no cooperativity, is also considerable here. Thus, in construct λEwt 101 also (Figure 5, line G), both real repression and deactivation contribute to apparent repression. What if O3 is now restored to its natural position and combined with another O3 at the position of O1 (λEwt 303, Figure 5, line F)? Cooperativity can still be observed.

It is interesting to note that while each O3 alone exerts no measurable effect (λEwt 300, Figure 4, line E and λEwt 003, Figure 5, line D), repression by the combination of both (38-fold) is even better than the effect of one O2
in the absence of auxiliary operators (21-fold). Recall that the affinity of O2 for repressor is ~30 times higher than that of O3. With high amounts of repressor, cooperativity does not occur. Repression is not much higher than the product of repression by λEwt 300 (Figure 4, line E) and deactivation by λEwt 003 (Figure 5, line D).

No cooperativity over long distances
If cooperativity between operators works exclusively by local increase of repressor concentration, one would not expect to observe an interaction between even completely occupied operators, if they lie too far apart. To test this assumption for the lac system, we inserted an ideal lac operator behind the lacZ gene at a distance of ~3600 bp from O1 (λEwt 100i, Figure 6, line B). Figure 6 shows that this additional operator has very little, if any, effect on repression.

Discussion

DNA binding and cooperativity
Many transcription factors enhance their specificity and strength of DNA binding by aggregating to dimers (Freemont et al., 1991). The monomers touch each other with the same surface and the DNA binding motifs come to lie adjacent to each other. This is then reflected in palindromic target sites. The two subunits cooperate with each other in binding to the two target half-sites. The mechanism of this cooperativity can be described as an increase in local concentration of the second monomer (Schleif, 1988). Once one of the subunits has properly bound to one target half-site with the association rate $R_1$, the other subunit is brought into correct orientation extremely close to the second half-site. This proximity determines the extremely high association rate $R_2 >> R_1$.

In an analogous manner, one can envisage how the extensive network of contacts between one protein monomer and its target (Pabo and Lewis, 1982; Pabo and Sauer, 1984; Chuprina et al., 1993) could have evolved. Once two strong interactions exist, the orientation of the protein along the DNA is fixed and every additional contact strengthens the complex, ideally with its whole energy. The interacting functional groups will be brought into proper orientation close together.

The increase of local concentration
Here, we would like to outline the idea of a local increase of repressor concentration. This discussion does not take into account the constraints exerted by the DNA between two operators.

The possibility of locally increasing the concentration of repressor with respect to an operator by DNA loop formation depends on both the quality and position of the auxiliary operator, as well as on the cellular concentration of repressor. Closure of the loop takes place when the free binding region of a DNA-bound tetrameric repressor molecule has a higher concentration with respect to the second target site than the repressor free in solution. A repressor molecule bound to an auxiliary operator is, with respect to a second operator, trapped in a sphere whose radius is given by the length of the DNA between the two operators. The quotient of fractional occupancy of the auxiliary operator with the volume of the sphere in which it is trapped divided by the quotient of the number of repressor molecules per cell with the cell volume gives the cooperative increase of occupancy of the other operator.

DNA looping
If a dimeric DNA binding protein gains the ability to form tetramers, it will be able to increase its binding strength and specificity by cooperativity. Lac repressor dimers gained the capability to form tetramers through the addition of two suitable leucine heptad repeats to their C-termini (Alberti et al., 1991). They are then capable of forming a four helical bundle (Alberti et al., 1993). Since the Lac repressor tetramer can bind independently to two operators (Kania and Müller-Hill, 1977; O’Gorman et al., 1980; Culard and Maurizot, 1981; Krämer et al., 1987, 1988), cooperative binding will only depend on whether binding of one dimer brings the second dimer close enough to a second target site. Since the two DNA binding regions of the tetramer point in opposite directions [the angle between two bound DNA molecules is unknown, but electron micrographs (Krämer et al., 1988) seem to indicate that they are not parallel], the protein or the DNA have to bend to enable the second dimer to reach the additional target. This requires energy and determines the maximal proximity of the second binding region of the protein and the additional DNA target. The interacting partners are pre-oriented, at least over short distances, by the DNA which lies between the target sites. Both the amount and direction of a possible intrinsic curvature and the stiffness of the intervening DNA depend on its sequence. For effective loop formation, the target sites must lie on the same side of the DNA. Cooperative interaction between two lac operators and wild-type Lac repressor occurs.

![Diagram of repression by Lac repressor](https://example.com/diagram.png)

Fig. 6. Repression values for chromosomal (see Materials and methods) lacZ genes. Repression values are given as the specific β-galactosidase activity in the absence of active Lac repressor divided by the specific activity in the presence of the indicated Lac repressors. For details see legend to Figure 3. A, λEwt 100 (from Figure 3); B, λEwt 100i.
The reality of the lac system

Table I lists the occupancy of O1, O2, O3 and the ideal lac operator in vivo in the presence of five times and 90 times wild-type amounts of tetrameric or dimeric Lac repressor. The values are calculated from the repression factors of Figure 3. Their relatively low affinity for Lac repressor and their distance from the lac promoter caused the belief that the lac auxiliary operators O2 and O3 have little or no impact on repression of the lac operon (Pfahl et al., 1979). At low concentrations of Lac repressor, they indeed do not work efficiently in blocking polymerase at the position of O1 (Figure 3), but together with O1 they exert a large cooperative effect from their natural positions (Figure 4, line B and Figure 5, line B). With wild-type amounts of Lac repressor (1×10^-8 M; Gilbert and Müller-Hill, 1966), each of the auxiliary operators enhances repression by O1 to about the same extent (Oehler et al., 1990). The quality of the auxiliary lac operators corresponds inversely to their position relative to O1. O2 binds Lac repressor just ~10-fold less well than O1 and is separated from O1 by 401 bp. O3 lies at a distance of only 92 bp from O1 (Figure 1), but its affinity for Lac repressor is about another 30-fold lower than that of O2. This observation fits into the model of cooperative repression by DNA loop formation. At a concentration of 50 molecules of tetrameric Lac repressor per cell (2×10^-12 cm^3; see Figure 7), there is on average one molecule per 4×10^-14 cm^3. If one molecule of repressor is bound to O2, it is trapped in a volume of 1×10^-14 cm^3 around O1 (Figure 7). Neglecting the resistance of the intervening DNA to bending, the Lac repressor concentration should be correspondingly elevated with respect to O1. Experimentally, we observe an ~10-fold increase of repression by an operator at the position of O1 through addition of O2 (Figure 4).

A bad operator (O3), which is not frequently occupied, has to lie very close to O1 in order to enhance repressor concentration. While at the i+ concentration of Lac repressor, O3 is ~10% occupied, occupancy at five times i+ is ~50% (Table I). One Lac repressor tetramer bound half of the time to O3 gives an average concentration of one molecule in 2×10^-16 cm^3. Lac repressor concentration with respect to O1 is then 200-fold above the cellular concentration. Experimentally, with five times i+ amounts of tetrameric Lac repressor, an ~30-fold increase of repression by O1 upon addition of O3 is observed (λEwt 100, Figure 3, line A and Figure 5, line B). This discrepancy fits with the fact that a relatively high amount of energy is needed to bend a 92 bp DNA, which reduces the effective local concentration of Lac repressor. The length of the Lac repressor molecule that connects the two operators should reduce the extent to which the short intervening DNA has to bend. But the orientation of the two DNA binding regions of Lac repressor is not known. Thus, it is not clear if the Lac repressor tetramer really decreases the extent to which the DNA has to bend or asks for simultaneous bending of the intervening DNA in two directions.

We could not find cooperativity between operators separated by a distance of ~3000 bp, where no increase of local Lac repressor concentration is expected. In contrast, at the distance of 92 bp, even two O3 operators, which individually lead to no detectable effect, repress the lac promoter efficiently with five times i+ amounts of wild-type tetrameric Lac repressor (Figure 5, line F). A similar cooperation has been reported for synthetic weak lac operators (Amouyal and von Willecken-Bergmann, 1992). O2 enhances repression by O1 ~10-fold at five times i+ amounts of Lac repressor. This factor corresponds to a local increase of Lac repressor concentration relative to O1 or any operator in its place. Thus, in a background of ~20-fold more cellular Lac repressor, the repressor molecule bound to O2 should exert a barely detectable effect. This is exactly what is observed (Figure 4).

A simple model for DNA loop formation as the basis of a locally increased concentration for Lac repressor is given in Figure 7. What our model does not account for is the observation of interactions between target sites for transcription factors over long (>1000 bp in prokaryotes) or very long (>100 000 bp in eukaryotes) distances. It will be interesting to know how the topology of the out-looping DNA or the action of multiple binding sites can make this long-range cooperation possible.
Materials and methods

Chemicals, media and enzymes
[14C]Deoxyribonucleoside triphosphates were obtained from Amersham Buchler (Braunschweig, Germany); 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was from Bachem fine chemicals (Torrance, USA); ATP, deoxyribonucleoside triphosphates, dithiothreitol, ethidium bromide and ‘Trisma base’ from Sigma Chemie (München, Germany); β-mercaptoethanol from Fluka AG (Buchs, Switzerland); o-nitrophenyl-β-D-thiogalactoside (ONPG) from Serva Feinbiochemie (Heidelberg, Germany); area from Bethesda Research Laboratories (Neu-Isenburg, Germany); and the chemicals for automatic DNA syntheses from Applied Biosystems (Pflugstadt, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) or Sigma Chemie (München, Germany). Agarose, acrylamide and N,N'-methylenebisacrylamide were obtained from Bethesda Research Laboratories (Neu-Isenburg, Germany).

All enzymes were purchased from New England Biolabs (Bad Schwalbach, Germany) or Boehringer (Mannheim, Germany). 77 polymerase sequencing kits were obtained from Pharmacia (Freiburg, Germany). Oligonucleotides were synthesized by Karin Otto and Gudrun Zimmer on an Applied Biosystems Synthesizer model 394 and were purified on denaturing polyacrylamide gels.

Bacteria, plasmids and λ phages
E. coli strain BMH 8117 F' has the genotype: (lac pro naI supE F' lac Z Y pro A B'). The epiphasme has been described by Gho and Miller (1974). It carries an I-Z deletion, leaving the 5' end of the I gene intact.

Plasmids were constructed according to standard procedures (Maniatis et al., 1982). Plasmid pS0 1010-P1 codes for about five times 106 copies of wild-type tetrameric Lac repressor, plasmid pS0 1000 codes for 90 times 106 copies of Lac repressor, plasmid pS0 1000 AA carries an I' allele. These plasmids have been described previously (Oehler et al., 1980). Plasmid pS0 331 Stop codes for about five times 106 copies of dimeric Lac repressor and plasmid pS0 3310 Stop codes for 90 times 106 copies of dimeric Lac repressor. These plasmids were derived from plasmids pS0 1010-P1 and pS0 1000 respectively. A SpeI restriction site has been introduced in codons 329 and 330 (Alberti et al., 1991) by site-directed PCR mutagenesis (Kadowaki et al., 1989). This led to an exchange of amino acid 330 from Leu to Ser. Then, a synthetic piece of DNA was cloned between the SpeI site and a PstI site which is located C-terminally of the lacI coding sequence. The synthetic DNA inserts an ochre stop codon after codon 330. The resulting mutant protein is dimeric, since it lacks the 30 C-terminal residues of wild-type tetrameric Lac repressor, which carries the two leucine heptad repeats that enable Lac repressor dimers to aggregate to tetramers (Alberti et al., 1991, 1993). The pEwt plasmids carry a lacZ gene under control of the wild-type lac promoter and different combinations of lac operators. Plasmids pEwt 123, 103, 120, 100 and 003 have been described (Oehler et al., 1990). The numbers 1, 2 and 3 stand for the presence of the first, the second or the third lac operator respectively, whereas the 0 indicates the absence of a particular operator. The name of a particular operator is given by its position. The first number designates the natural position of the first lac operator (O1), the second number the natural position of the second operator (O2) and the third number the natural position of the third operator (O3). Thus the combinations X00, X0X and XX0 indicate the presence of only one operator X at the position of O1 or O2 or O3 respectively. Plasmids pEwt 200, 220, 300, 320, 301, 303, 101, 103 and 001 were generated by PCR mutagenesis of the respective wild-type plasmid. Plasmid pEwt 100 was constructed by the insertion of a 20 bp synthetic ideal operator into the unique AarII restriction site behind the lacZ gene of pEwt 100, at a distance of ~3600 bp from O1. To introduce the ideal lac operator into λacist 100, we first replaced O1 with a SpeI restriction site. Then, a double-stranded synthetic 20mer encoding the OId, with compatible 5' protruding ends, was cloned into the SpeI site. The introduction of the SpeI site lowered the expression from lac promoter >20-fold. Repression values higher than 4000-fold could not be reliably determined for λacist 100. The Xbal fragments that contain the lacZ gene and the ampillin resistance gene, but neither the tetracycline resistance gene nor the pBR-derived origin of replication, were isolated and ligated into the unique Xbal restriction site of phage λP1 (Siegel et al., 1989) to generate the respective λEwt phages. In vitro packaging of λ DNA and infection of host cells were performed according to standard procedures (Maniatis et al., 1982). Lysogenic colonies were isolated and checked on X-Gal indicator plates for stable integration of the Z' prophage. For each construct, several lysogenic isolates were tested for constitutive β-galactosidase expression. Sporadic isolates with

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References


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