MINIPREPARATION OF PLASMID DNA

Introduction

In this lab period, we will begin to determine whether we have any recombinant plasmids (positive clones). First, we will isolate plasmid DNA from two of the transformed bacteria. Next we will cut the recombinant plasmid with the appropriate restriction enzymes. After DNA digestion we will load an agarose gel and the results will be examined during our next lab period.

The minipreparation of plasmid DNA, or **miniprep**, is a *small-scale isolation of plasmid DNA from bacteria*. Two main approaches have been used for this isolation; the first and more traditional technique is often a variation on the original protocol by Birnboim and Doly (1979). The second uses a special silica matrix to bind DNA and then release it under certain conditions. The matrix is often included in a spin-column. Many of the biotechnology companies sell kits for plasmid isolation that use this technology. We will use the traditional method for the miniprep and you will have an opportunity to use the spin-columns in other isolation procedures.

LEARNING GOALS:

    1. Understand the biochemical and molecular effects of each reagent used in the miniprep protocol.
    2. Understand the difference in mobility between a DNA fragment and an intact plasmid.
    3. Know the main conformations of uncut plasmid DNA and be able to recognize them on a gel.
    4. Be able to recognize the major differences between bulk prep plasmid DNA (which is very pure) and miniprep plasmid DNA.

Topic I: MINIPREPARATION OF PLASMID DNA

A single colony is taken from the LB-Amp/X-gal transformation plates and used to inoculate 1 mL of LB-Amp broth (Should we use a blue or white colony?). For the miniprep procedure, bacteria should be in **stationary phase** so the tube is incubated with shaking overnight at 37° C to obtain a saturated culture. In a series of a few simple steps with five reagents, plasmid DNA is separated from the cellular proteins, lipids, and plasmid DNA. The miniprep yields 2-5ug of plasmid DNA.

However, the purification is rather crude and may contain small pieces of chromosomal DNA. Rough pipetting can break off pieces of chromosomal DNA (**shearing**). Shearing will also break the supercoiled plasmid, producing the **nicked circle** or even the **linear conformations** and reducing the amount of desired **supercoiled conformation**. There may be traces of **nucleases** (DNase) that will degrade DNA and shows up as smearing in the gel lane. Finally, salts and other impurities will reduce the activity of restriction enzymes. Thus the miniprep is likely to have **partial digests** of the plasmid.

Separate procedures are used for large-scale or "bulk preps" of plasmid DNA. These protocols may start with 25 - 1000 mL of bacterial culture.

Miniprep Solutions

1. **(GTE) Alkaline Lysis Solution I**

50 mM glucose   25 mM Tris-Cl (pH 8.0)   10 mM EDTA (pH 8.0)
  Glucose acts to maintain osmotic pressure, and the Tris buffers the cell at pH=8.0. The EDTA binds divalent cations in the lipid bilayer, which weakens the cell envelope. After cell lysis (next steps) EDTA limits DNA degradation by binding (chelating) Mg^{++} ions that are a needed cofactor for bacterial nucleases.

2. **(SDS/NaOH) Alkaline Lysis Solution II**

caution 0.2 N NaOH (freshly diluted from a 10 N stock)   caution 1% (w/v) SDS
  This alkaline mixture lyses the cells.
  The SDS detergent dissolves the lipid components of the cell membrane and cellular proteins. Sodium hydroxide denatures both the chromosomal and plasmid DNA into single strands; the two strands of intact plasmid DNA remain intertwined.

3. **(3 M KoAC/Acetic Acid) Alkaline Lysis Solution III**

5 M potassium acetate, 60.0 ml   caution glacial acetic acid, 11.5 ml
H_2O, 28.5 ml
  The acetic acid solution brings the pH to neutral, and the DNA strands can renature. The large chromosomal strands cannot rehybridize perfectly, but instead become a partially-hybridized tangle. Potassium acetate precipitates the SDS (with its lipids and proteins) from the solution. The SDS/lipid/protein precipitate traps the tangled chromosomal DNA. This creates the "white goop" that pellets after centrifugation. Only the plasmid DNA, small fragments of chromosomal DNA, and RNA remain in solution.

4. **Isopropanol**
  This alcohol rapidly precipitates nucleic acids. However, if allowed to sit for longer, proteins will also precipitate. Thus we time this step for a quick precipitaion before centrifugation.

5. **Ethanol**
  An ethanol wash helps remove salts and any remaining SDS as these can interfere with a restriction digest.

6. TE buffer

10 mM Tris-Cl (desired pH)   1 mM EDTA (pH 8.0)

Tris buffers the DNA solution. EDTA binds divalent cations (especially Mg^{++} ions) that are a needed cofactor for bacterial nucleases and thus limits DNA degradation.

Topic II: RESTRICTION DIGESTION TO IDENTIFY POSITIVE CLONES

Interpreting the gels showing plasmid DNA is not always straightforward and requires practice and the "dirtier" miniprep DNA can further complicate the interpretation.

1. Movement of DNA Fragments

Pieces of DNA, for example DNA fragments cut by restriction enzymes, separate in an agarose gel according to size. The smaller pieces travel the fastest and the farthest through the gel. The larger pieces have more difficulty moving through the gel matrix and thus move more slowly through the gel. However, *the movement of DNA fragments in the gel is logarithmic and not arithmetic*. This means that the smaller (lighter) fragments move faster than one would expect and the larger (heavier) fragments move even slower than one would expect if the relationship were arithmetic. When we use a standard curve of known fragment sizes to determine the size of fragments of unknown length, the <u>distance migrated</u> is plotted on an arithmetic scale (in millimeters or centimeters traveled). But he size or <u>mass of the fragment</u> is plotted on a **log^{10} scale**.

Examine Figure 5.1 on the next page. Note the small distance between the fragments of 6527 base pairs (bp) and 5505 bp, even though there is a 1000 bp difference between the two. Now look at the distance separating the 784 bp and 564 bp fragments. These fragments are more widely separated than the larger fragments, even though the size difference between the smaller fragments is only 220 bp.

2. Fragment Resolution

There are limits to the ability of agarose to separate DNA fragments of different sizes. When two fragments are close together in size, they may appear as one band in the gel rather than two. For example; if you expect 6 DNA fragments and thus 6 bands on a gel but you see only 5 bands, then it is likely that two of the fragments have not **resolved** in the gel. It is termed a **doublet** when two fragments appear as one band. Carefully examine Figure 5.1 and notice that there are two doublet bands.

3. Conformations of Uncut Plasmid DNA

We analyze DNA that has been cut with restriction enzymes by determining fragment sizes using agarose gel electrophoresis. However, *uncut* plasmid DNA has several distinct **conformations** that can be identified when the uncut plasmid is electrophoresed in an agarose gel.

A) Supercoiled DNA (Form I), is the fastest conformation of the uncut plasmid. The enzyme DNA gyrase introduces these extra twists into chromosomal and plasmid DNA of bacteria. The bacteria use the superhelical tension to assist in processes

like replication and transcription. The isolated supercoiled (**sc**) plasmid DNA is wound up into a compact structure. Imagine taking a circle of string and rolling it around in your hands until it forms a little ball. Because of its compact shape, sc DNA is the **fastest** moving conformation in the gel.
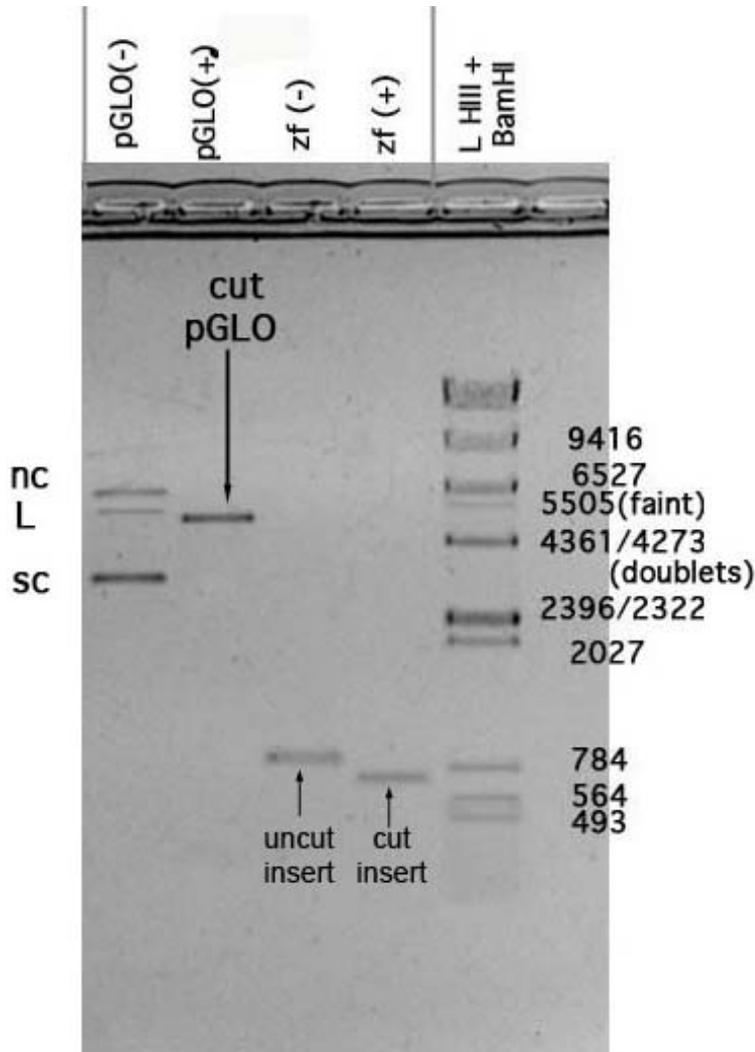

Figure 5.1: A ladder lane that contains two doublets. The ladder is a *Hind*III+*Bam*HI digest of Lambda. This Figure also gives examples of the conformations of uncut plasmid DNA in the lane marked pGLO(-). This is not miniprep DNA and the lanes are clean.

B) Nicked Circle DNA (Form II) is also called relaxed circle. In bacteria, the enzyme topoisomerase I will nick one strand of the helix so that DNA polymerase has access to the DNA for replication. Once one of the strands has been cut, the superhelical tension relaxes and the tightly-wound ball becomes a floppy circle. A nick can also occur during isolation of the plasmid because of enzyme activity or mechanical shearing of the DNA. Nicked circle (**nc**) is the **slowest** conformation of uncut DNA.

C) Linear DNA (Form III) is produced when a restriction enzyme cuts a plasmid at only one site. Both strands of the helix are cut at the same place. Linear DNA can occur because of endonuclease contamination of the isolated plasmid, or because of harsh treatment. On a gel the linear DNA will run **between** the sc and nc conformations (possibly closer to the sc band).

These are the three main conformations of single plasmid molecules, or **monomers**. However, under certain conditions, two plasmid molecules can join to form a **dimer**. Since the dimer form is twice as large as the monomer, it will run higher in the gel. It is possible to have sc, nc, and linear forms of the dimer. As with the monomeric plasmid, the sc will be the fastest conformation and the nc will be the slowest. It is also possible for more than two plasmid molecules to join and create high molecular weight **multimers**. It is usually difficult to distinguish conformations of multimers. We will concentrate on single plasmid molecules.
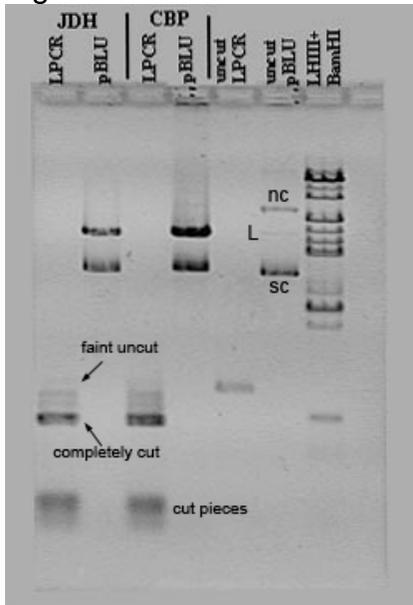
Figures 5.2 - 5.4 show several examples.

Figure 5.2: In this cloning experiment, a fragment of Lambda DNA (LPCR) is inserted into the plasmid pBLU. The concentration of uncut pBLU (lane 6 from left) is low, so the linear conformation is faint.
The LPCR fragment has small pieces cut from the ends. The first four samples from the left are student minipreps.
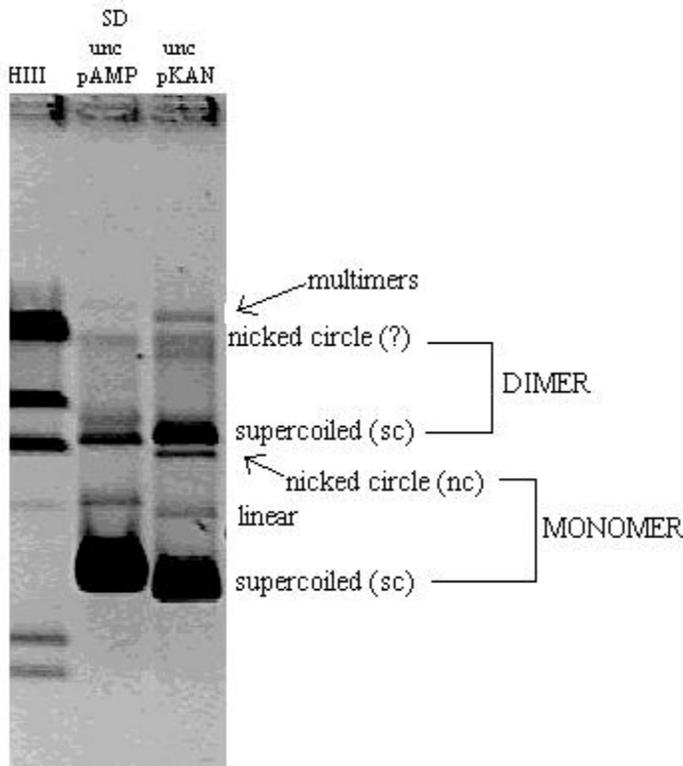

Figure 5.3: Very overloaded lanes of uncut plasmids pAMP and pKAN (how do we know these lanes are overloaded?). However, because there is so much DNA, all of the conformations are visible. HIII is a Lambda *Hin*dIII digest used as the ladder.
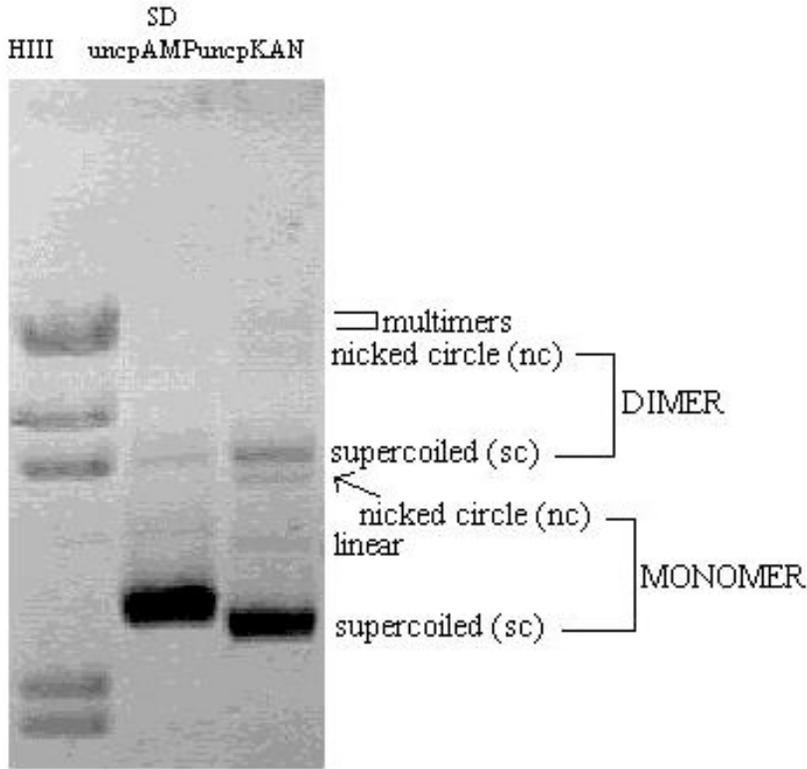
Figure 5.4: Another example of the conformations of uncut plasmid DNA. Which plasmid is larger, the pAMP or the pKAN? How can you tell?
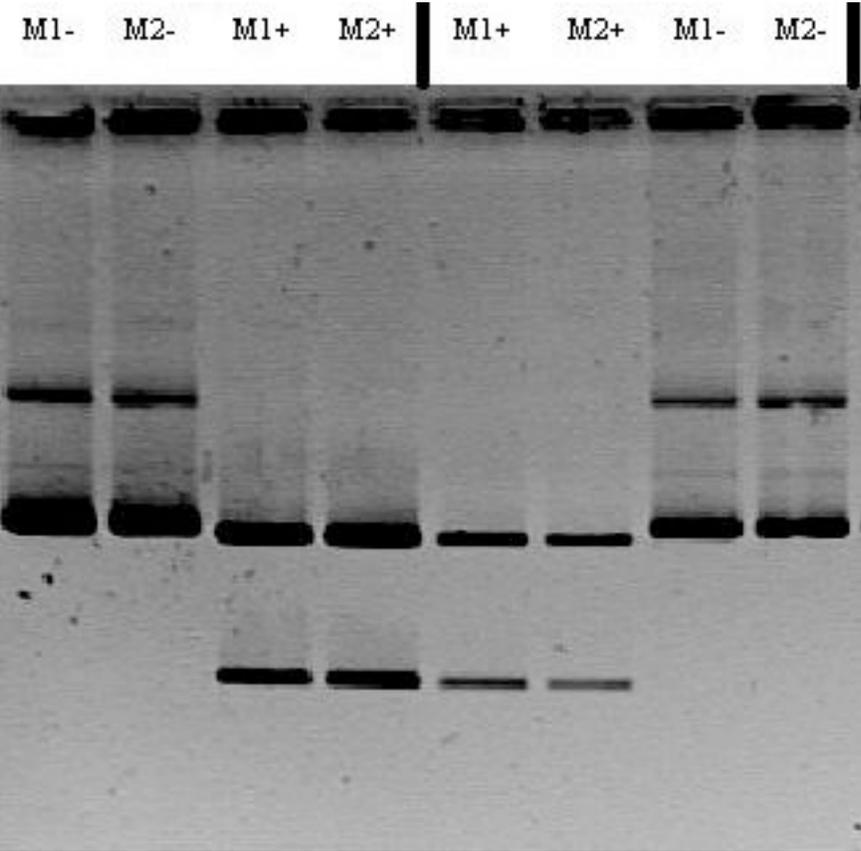

Figure 5.5: Several samples of miniprep DNA. M1 and M2 stand for miniprep one and miniprep two, respectively. The (+) or (-) represent WITH or WITHOUT restriction enzymes. Note the dark wells and the smear in some of the lanes. But overall, these are very good minipreps and all the minipreps show complete digests.

4. Small-scale and Large-scale Preparation of Plasmid DNA

Depending on the need, plasmid DNA can be isolated in different quantities. Small-scale preparation of plasmid DNA is often termed a "miniprep" and starts with 1-5 mL of an overnight bacterial culture. There are many different miniprep protocols available. Several micrograms of DNA can be isolated with the miniprep, and the procedure can be completed in a relatively short time. Minipreps are often used to detect successful recombinants after the potential recombinant DNA has been inserted into bacteria (the process of transformation).

Many micrograms of DNA can be isolated using a large-scale or "bulk prep" protocol. These protocols use from 25 to 1000 ml of bacterial culture. Formerly, large-scale isolation of plasmid DNA required many hours of ultacentrifugation in a solution of cesium chloride-ethidium bromide. This procedure was time-consuming and required handling of toxic materials. Now, the same resins that can be purchased commercially for the miniprep are commonly employed for bulk preparation of the plasmid DNA and the procedure can be completed in several hours.

5. Differences Between Bulk Prep and Miniprep DNA

While all the bulk prep procedures result in high-quality plasmid DNA if performed properly, there are often differences in the quality of miniprep DNA. The miniprep may contain pieces of sheared chromosomal DNA and various proteins. Minipreps usually contain RNA, so RNase is added at some point during purification. Finally, there may be salts or traces of the miniprep reagents in the isolated DNA.
When is RNase added in our protocol? _____
Which samples receive the RNase? _____

Because of these differences in purity, the miniprep DNA looks and cuts differently than the bulk prep plasmid. Miniprep DNA can be more difficult to cut because associated proteins may interfere with recognition of the cleavage site, and contaminants can result in sub-optimal reaction conditions in the restriction digest. Thus, it is more common to see a **partial** or **incomplete digest** with miniprep DNA. The RNA is able to bind ethidium bromide. If it is not degraded by addition of RNase, it will form a diffuse "**RNA cloud**" towards the bottom of the gel. If you are trying to detect a small fragment from a restriction digest, it can be obscured by the RNA cloud. Finally, the gel lanes of miniprep DNA are often dark or smeared. Examine the two gel pictures in Figure 5.5 and the "RNA cloud" in figure 5.6. This contrasts with the clear wells and gel lanes of bulk prep DNA, except for the presence of discrete DNA bands.

Questions to ponder:

1. Explain why EDTA is an important component of TE buffer, which is used to dissolve the miniprep DNA.

2. Compare the lanes containing your miniprep DNA with the lanes containing control plasmid. Explain the possible reasons for variations.

3. Identify the conformations of uncut plasmid in the control lane and in your uncut miniprep lanes.

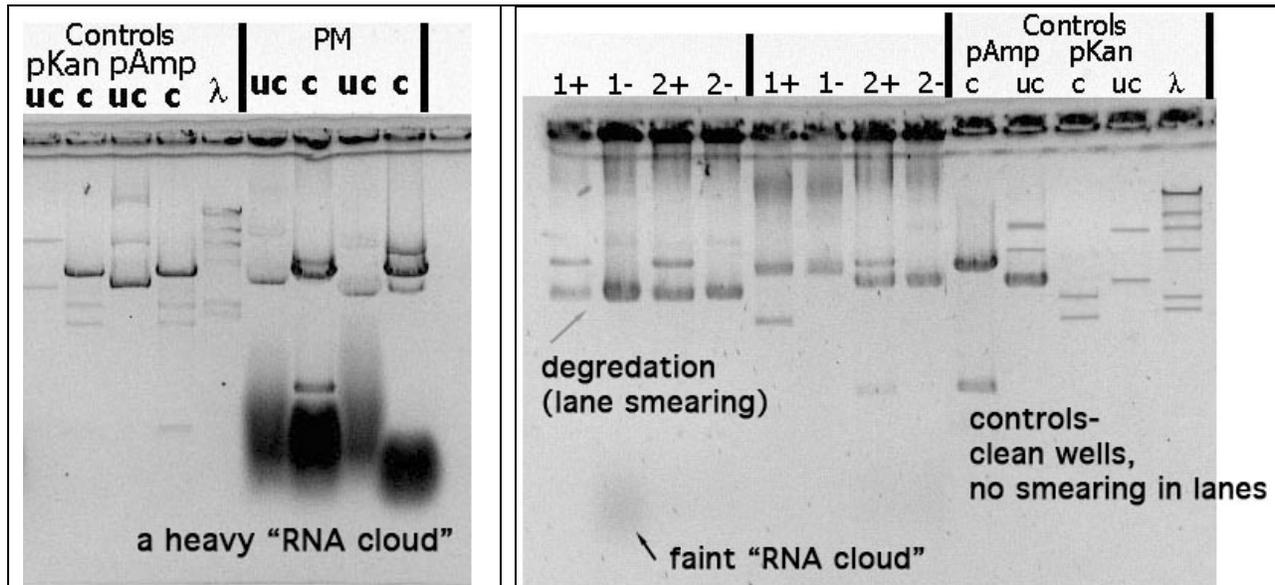4. Were any positive clones found in your class gels? *How can we identify a positive clone?*

Figure 5.6: Comparison of miniprep with bulk prep DNA. (left) Control DNA of uncut (UC) and cut (C) pAMP and pKAN plasmids. Note that the control DNAs are partial digests! The samples from student PM did not get RNase, and there is a heavy RNA cloud that can obscure any DNA bands in the area.
(right) The first eight lanes are two sets of student minipreps, where the numbers 1 and 2 represent the two miniprep samples with (+) or without (-) restriction enzyme. These lanes show the smearing that can be found in miniprep DNA from digestion by DNase, and some of the wells appear darker from pieces of chromosomal DNA. One of the minipreps still has a very faint RNA cloud but this is not significant. Compare the control DNA with the miniprep DNA – no smearing in the lanes and no dark staining in the wells. Did the controls cut to completion in this experiment? How can you tell?

REFERENCES

Birnboim, H.C. and Doly, J. 1979. A rapid alkaline procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513–1523.
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