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Developing Breast Cancer? A Molecular Epidemiological
Study

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13. ABSTRACT (Maximum 200 Words) In a case-control study, nested within a cohort of 4,888 women with BBD, we demonstrated that p53 protein accumulation detected by immunohistochemistry was associated with a 2.5-fold increase in the risk of subsequent breast. However, by using immunohistochemistry alone, we may have underestimated the true risk of developing breast cancer. We hypothesized that p53 mutations in benign breast tissue are associated with increased risk of subsequent breast cancer. We are testing our hypothesis by: (1) analyzing benign breast tissue from 138 cases and 556 controls for the presence of p53 mutations using PCR-SSCP and PCR-direct DNA sequencing; and 2) estimating the risk of breast cancer in relation to: (a) the presence of p53 mutations in BBD; and (b) the presence of both p53 mutations and p53 protein accumulation in BBD. We also propose to compare mutations detected in the cancers with those detected in their preceeding benign breast tissue samples. In the first year of the grant, we have established the conditions for the PCR-SSCP for exons 2 to 11. 126 subjects have had sections cut, epithelium enriched tissue microdissected out, and DNA extracted. The DNA has undergone PCR-SSCP under two conditions and those with abnormal gel patterns have undergone repeat PCR-SSCP. Abnormal migrating bands have been cut from the gel. These will be sequenced. We have determined that manual sequencing rather than automated sequencing is more appropriate to analyze the p53 gene in these small paraffin embedded benign lesions.			
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INTRODUCTION

Our work is directed towards identifying genetic and protein changes in benign breast disease (BBD) which might be involved in the pathogenesis of breast cancer, and which might serve as markers of risk. We have recently completed a case-control study, nested within a cohort of 4,888 women with BBD, in which we demonstrated that p53 protein accumulation detected by immunohistochemistry was associated with a 2.5-fold increase in the risk of subsequent breast cancer (1). However, by using immunohistochemistry alone, we may have underestimated the true risk of developing breast cancer in association with p53 changes since approximately 33% of p53 mutations do not alter the protein in such a way that there is positive immunostaining (2-5). Therefore, a more complete assessment of the role of the association between p53 and breast cancer risk will come from studies combining both immunohistochemistry and p53 gene sequencing. We hypothesized that p53 mutations in benign breast tissue are associated with increased risk of subsequent breast cancer.

We are testing our hypothesis by:

- (1) analyzing benign breast tissue from 138 cases and 556 controls for the presence of p53 mutations using PCR-SSCP and PCR-direct DNA sequencing; and
- 2) estimating the risk of breast cancer in relation to: (a) the presence of p53 mutations in BBD; and (b) the presence of both p53 mutations and p53 protein accumulation in BBD. We will localize the mutations to determine whether they occur preferentially in specific sites of the DNA and to compare them to known mutations listed in p53 mutation banks (6-9). We also propose to compare mutations detected in the cancers with those detected in their preceding benign breast tissue samples.

BODY

Task 1: Extracting DNA from paraffin blocks (Months 1-24):

- A) Cut histological sections from paraffin embedded tissue.
- B) Extract DNA

Task 2: PCR-SSCP analysis (Months 4-34):

- A) PCR for exons 2-11
- B) SSCP gels for each exon
- C) Autoradiography

We currently doing both task 1 and 2. To date we have generated primers for exons 2 to 11 of the p53 gene. We have developed the optimal PCR conditions, e.g. temperature, cycle number, primer concentration, and magnesium concentration, for each exon. We have cut histological sections from paraffin blocks (which contain the tissue to be analyzed), microdissected out the appropriate area in the tissue, and extracted the DNA using proteinase K from 152 subjects. We have examined the PCR products for each exon for 126 subjects. Each sample is run under two conditions (2 and 10% glycerol in

the loading buffer). Samples that show abnormal band migration in either one or both gels (please see a representative SSCP gel in the appendix 1) undergo repeat PCR. The new PCR product is then analyzed by repeat SSCP analysis. Those samples that show reproducible abnormal band migration are identified as samples that require DNA sequencing to confirm the presence of a p53 mutation or polymorphism. The bands are excised from the gels and stored at 4⁰C until they are further analyzed. By considering only those samples that show abnormalities in products from two separate PCRs as having gene alteration, we minimize the likelihood that the abnormality detected is an artefact resulting from nucleotide misincorporation during the PCR.

Task 3: Sequencing DNA with altered mobility on SSCP gels (Months 5-30):

- A) Excise band with altered mobility
- B) Elute DNA
- C) PCR appropriate exon(s)
- D) Sequence reaction and separation on acrylamide gels
- E) Autoradiography
- F) Automated sequencing

We are currently also working on task 3. Those samples that show reproducible abnormal band migration in the SSCP gels are identified as samples that require DNA sequencing to confirm the presence of a p53 mutation or polymorphism. The bands are excised from the SSCP gels and stored at 4⁰C until they are analyzed further. We are just beginning the next phase of eluting the samples and doing the sequencing.

We have compared the efficiency and reproducibility of automated sequencing to manual sequencing (part F). The Microgene Blaster™ (Visible Genetics, Toronto, CAN) which utilizes fluorescent labels was the automated sequencer used. This sequencer has been used successfully in other studies (10, 11). We obtained primers for exons 1 to 11 that were compatible with this sequencer. Using reagents obtained from Visible Genetics, sequencing of the PCR products was attempted. We found that if adequate amounts of DNA were used the p53 gene could be sequenced. However in our study we have only small amounts of DNA because unlike breast cancer, benign breast disease lesions are much smaller and thus we have much less starting tissue. We did detect base deletions and nucleotide changes in many of the samples examined but the alterations were not reproducible between analyses (see sequencing print out in appendix 2). All exons were examined to determine whether this problem was due to the method itself or specific to the exon and possibly the primers selected. All exons showed a similar lack of reproducibility. We concluded that for this study automated sequencing was not suitable. If possible we will examine whether the microarray technology might be more suitable.

KEY RESEARCH ACCOMPLISHMENTS

As we are just a year into the project for which the results are dependent on analysis of data from the entire case-control series, there are no key research accomplishments as of yet.

REPORTABLE OUTCOMES

There are no reportable outcomes as of yet.

Individuals who have been employed or paid from this grant include:

Melissa Cooper MSc student

ShuQiu Li Technician

Tajinder Bhardwaj Technician (started July 2000)

Hanje Chen Technician

Shudong Zhang Post Doctoral Fellow (2 months)

CONCLUSIONS

We are able to extract DNA from the paraffin embedded tissue samples and the DNA obtained is suitable for PCR-SSCP and sequencing.

Gene abnormalities detected by PCR-SSCP will have to be confirmed using manual sequencing. Automated sequencing is not sufficiently sensitive or reproducible when using the small amounts of DNA extracted from the paraffin embedded tissue obtained in this study.

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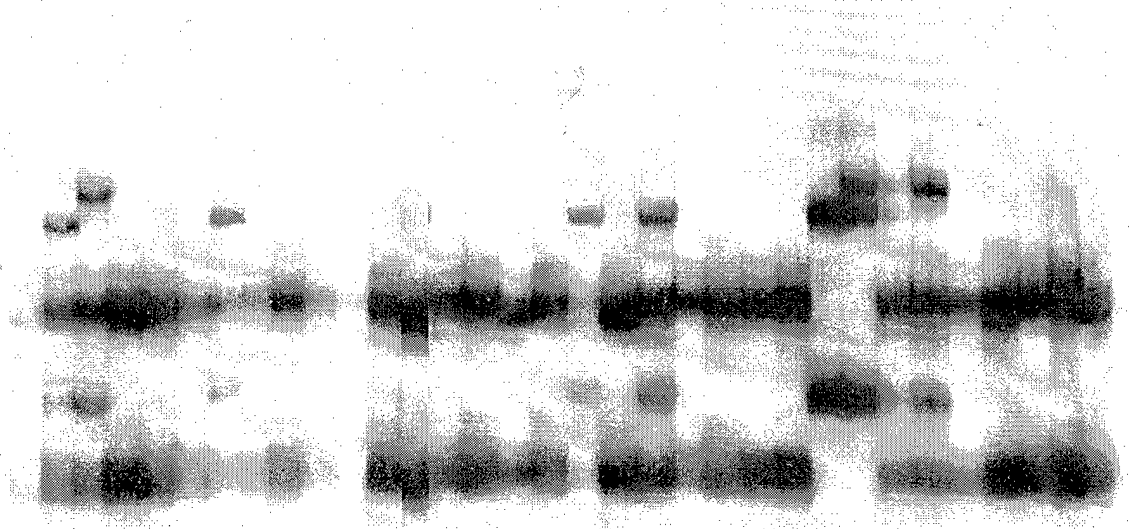
APPENDIX 1

PCR-SSCP GEL SHOWING 33 SAMPLES.

Altered mobility is seen in samples. The bands will be excised and the DNA sequenced to determine whether the sequence change represents polymorphism(s) or mutation(s). No PCR product was obtained for sample #92.

PCR-SSCP p53 EXON 3

31/ 53/ 55/ 57/ 58/ 65/ 70/ 72/ 79/ 83/ 92/124/126/175/187/188/189/190/191/192/195/196/197/199/201/203/205/206/207/208/220/222/224



APPENDIX 2

Sequences of exon 4 of the p53 gene obtained from DNA extracted from paraffin embedded breast tissue showing benign breast disease changes. The sample was run on two separate occasions (**A,B**) and different sequences were obtained.

BBD 36
3x5
EN-4

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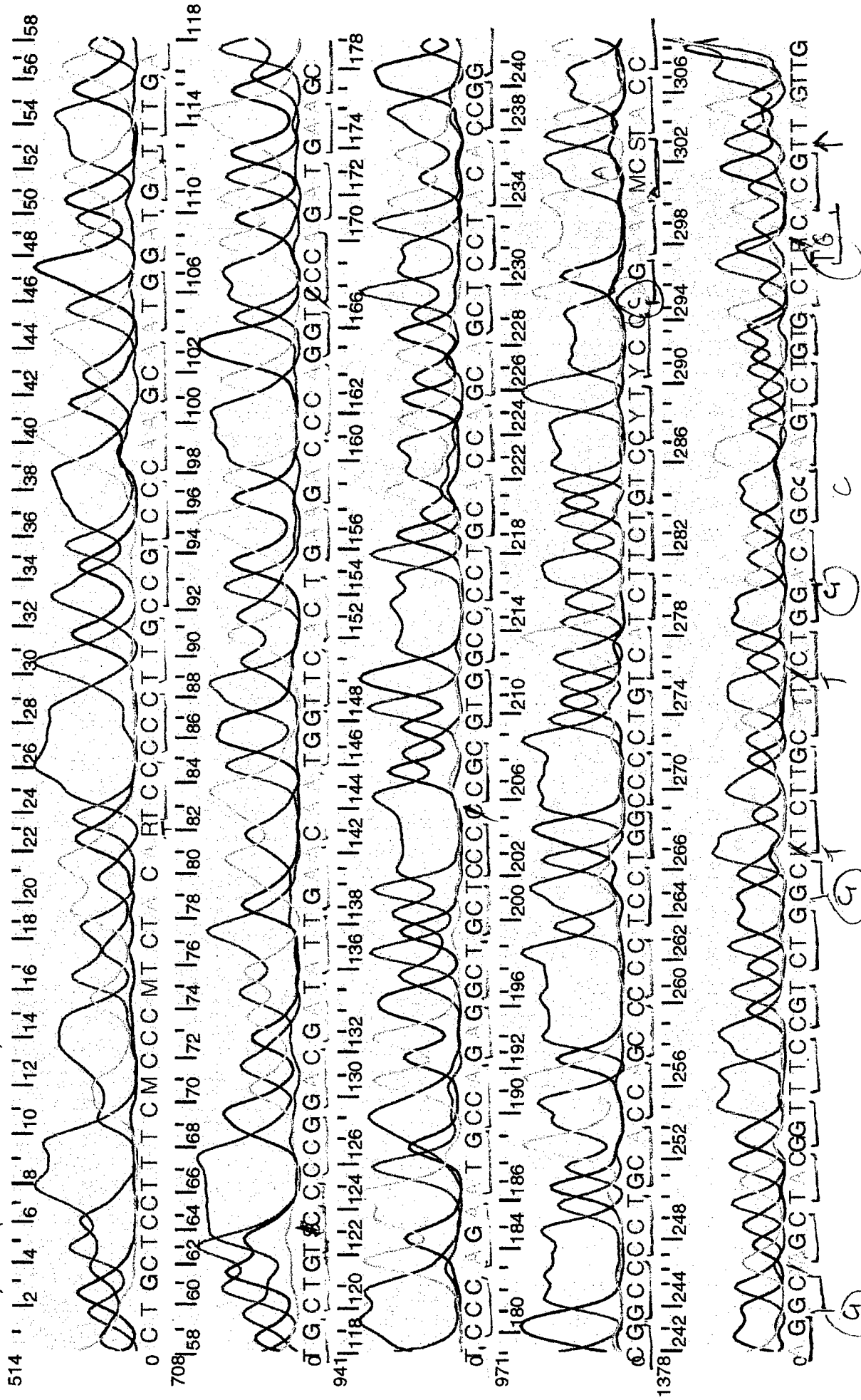
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Date: 20000126

Assay Notes: 26/1/2000

GeneObjects 3.1 (1999/07/13 20:28:45 EDT)





Date: 20000118

Assay Notes: PCR 13.1.2000

GeneObjects 3.1 (1999/07/13 20:28:45 EDT)

lab200001180945,1-4 - p53 Breast Tumors

Machine: 701098

Operator: Bhupinder

3x5
BBD 036

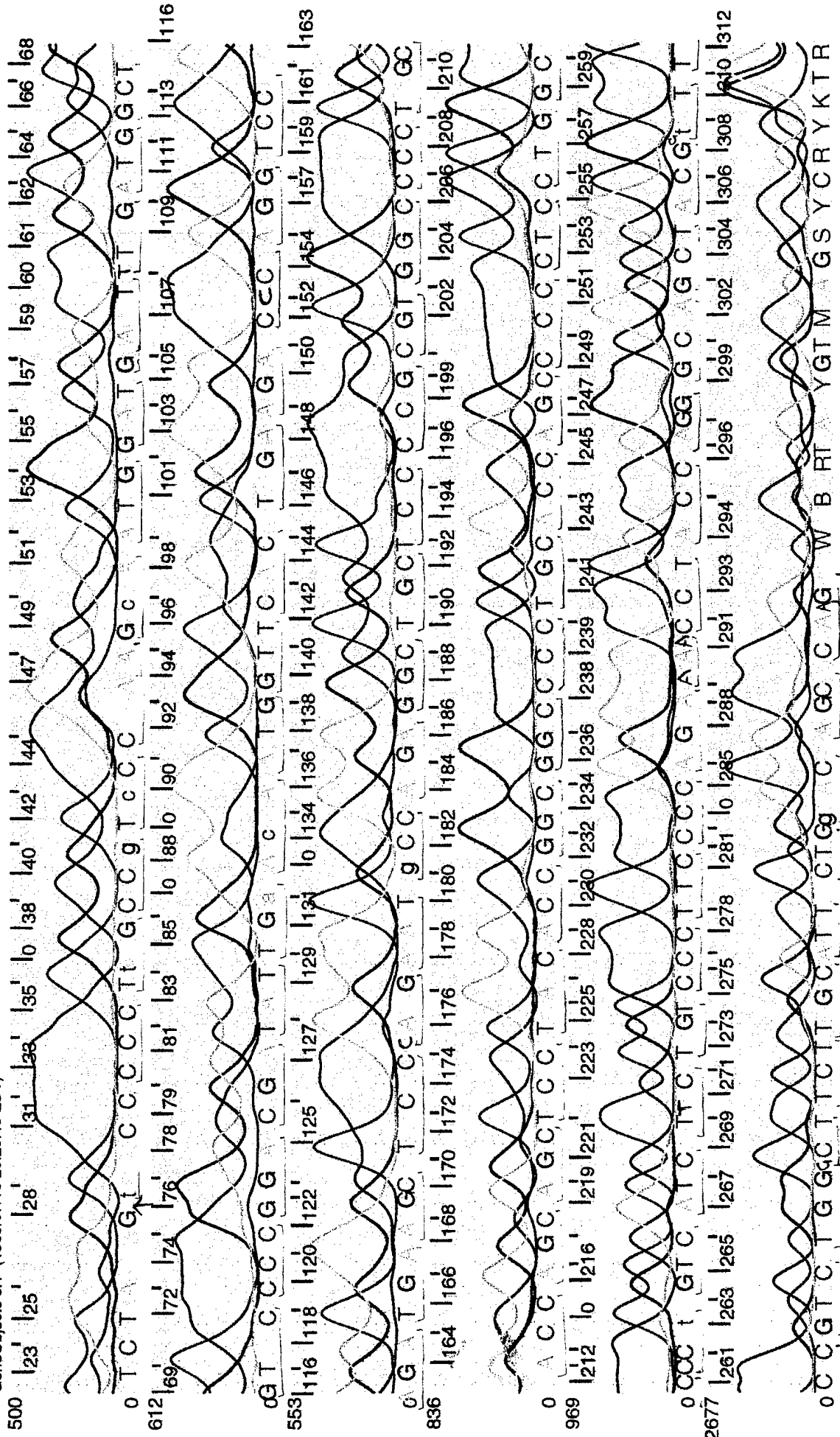


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