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DNA Polymerase Activity in Anterior
Pituitary Glands of Male Rats: Effects of Age
and Estrone Treatment

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Summary

DNA polymerase activity of the anterior pituitary glands of male rats was found to decrease with age in animals from 25 days to over a year old, and to increase after in vivo estrone treatment. These changes in activity correlate with changes in ^3H -thymidine incorporation into DNA under the same conditions of aging and hormone treatment.

The DNA polymerase activity was assayed by measuring the incorporation of ^3H -TTP into DNA in a medium containing Tris-HCl buffer (pH 8.5), the 4 deoxyribonucleoside triphosphates, Mg^{2+} , ATP and activated calf thymus DNA. About 75% of the DNA polymerase activity was found in the cytoplasmic fraction while 25% was associated with nuclei isolated from a tissue homogenate. The cytoplasmic activity showed the greater correlation with changes in the physiological state of the animal.

The isolated nuclei also retained endogenous DNA synthesizing activity in the absence of added

template DNA. The rate of synthesis remained unchanged with age or estrone treatment. Addition of cytoplasmic fraction stimulated activity by as much as 1.9 fold but stimulation was not correlated with the source of the cytoplasm. The response of nuclei to cytoplasmic polymerase as well as the fact that nuclei retained a DNA polymerase activity that could be measured with the activated DNA, suggest that the endogenous template is rate limiting in DNA replication in isolated anterior pituitary gland nuclei.

Introduction

Although cell turnover in the anterior pituitary gland is minimal there is good evidence that it fluctuates depending on the physiological state of the animal. From the results of numerous recent radioautographic studies¹⁻⁴ it is estimated that 1-2% of the pituitary cells in the glands of young adult rats are actively synthesizing DNA. In addition, another 1-2% will divide in response to a variety of stimuli. The class of cells which are invoked to divide appears to be closely correlated to a hormone-specific stimulus. For example, hormone imbalance resulting from either injection of estrogens or from castration results in increased DNA synthesis in acidophilic mammatrophs⁵ or in basophilic gonadotrophs^{6,7}, respectively. In addition to these changes brought about by manipulation of the animal, fluctuations in DNA synthesis normally occur diurnally⁸, during the estrous cycle⁹, and during pregnancy and lactation¹⁰. Finally, mitoses in the pituitary of the neonatal rat

are frequent but their incidence falls off by adulthood and continues to decline into old age^{6,8}.

We³ have previously shown that the rate of DNA synthesis in the incubated, intact anterior pituitary gland was comparable to that measured in vivo. The in vitro rate varied with age³, castration and photoperiod⁷. To further investigate the relationship between DNA synthesis and the secretory state of the gland we have measured levels and distribution of anterior pituitary gland DNA polymerase (deoxynucleoside triphosphate: DNA deoxynucleotidyltransferase, E.C. 2.7.7.7). In this study we report changes in the enzyme activity in the anterior pituitary glands of male rats of various ages and after estrone treatment. A preliminary report of this work has been given¹¹.

Materials and Methods

A. Animals

Male rats were obtained from the Holtzman Company, Madison, Wisconsin at least one week before use. They were kept on a 12 hour light, 12 hour dark cycle with feeding ad lib. Except where specified, the animals ranged in age from 33-55 days. Young (25-28 days) and old (8-15 months) animals were used in some studies. The old animals were classified by the Holtzman Company as "retired breeders". Their precise age was not specified by the supplier.

For some experiments, rats (46-51 days) were injected subcutaneously with estrone (National Biochemicals Co., 10 μ g in 0.15 ml corn oil). Other animals received corn oil only. Injections were given on alternate days over a period of 7 days, and the rats were sacrificed on the day after the final injection.

B. Preparation of cellular fractions

Rats were killed by cervical dislocation between 8:30 and 9:30 a.m. The pituitary glands were quickly

removed, and the pars distalis freed from the neural lobe and from most of the pars intermedia. This preparation of pars distalis (hereafter referred to as anterior pituitary gland) was homogenized in 0.25 M sucrose, 2 mM MgCl_2 (hereafter referred to as homogenizing medium) in a ground glass homogenizer. Depending on the age of the animal and the size of the pituitary, from one to four pituitaries were homogenized per 0.1 ml homogenizing medium. All preparations were carried out at 0-4°C unless otherwise noted.

Homogenates were centrifuged for 15 min (1000 x g, SS-34 rotor) in a Sorvall Superspeed RCS Refrigerated Centrifuge. The supernatant fraction was withdrawn and was used as the enzyme fraction in the cytoplasmic assays. Aliquots were either diluted with homogenizing medium or used directly to assay for DNA polymerase.

Nuclei were isolated from the pellet with a non-ionic detergent according to the method of Hymer and Kuff¹². The nuclei were resuspended in 1 ml homogenizing medium containing 0.1% Triton X-100 (Rhom and Haas

Co.), centrifuged, and washed twice in homogenizing medium. The final nuclear pellet was resuspended in homogenizing medium and used immediately for assay. Recovery of nuclei was approximately 70%.

Other nuclear isolation methods which involve centrifugation through different concentrations of sucrose were tried^{13,14}, but the yields of nuclei from the small amounts of pituitary tissue were too small (30-50%) to warrant use of these procedures.

C. Assay of DNA polymerase activity

DNA polymerase was assayed under conditions determined to be optimal for pH, Mg^{2+} concentration, and linear with respect to time and enzyme concentration. The incubation mixture contained in a final volume of 0.3 ml: 0.04 M Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 10 mM KCl, 1 mM 2-mercaptoethanol, 5 mM ATP, 0.2 mM each dATP, dCTP, dGTP (sodium salts, Sigma Chemical Co.), 0.15 mM [3H]-TTP (0.8-3.0 Ci/mM, obtained from New England Nuclear as 11.4-18.3 Ci/mM), 35 μ g calf thymus DNA, and cytoplasmic fraction (100 to 600 μ g protein). This reaction mixture is hereafter referred to as the

standard incubation medium. In some experiments nuclei (40-80 μ g DNA) were assayed in the same medium with or without cytoplasmic fraction or added DNA. Any additions or variations in the assay were made so that the final volume and concentration of components were unchanged.

Incubations were carried out in 12 ml conical glass tubes at 37°C with shaking. The reactions were terminated by placing the tubes on ice and adding cold 10% trichloroacetic acid. Three mg of Celite 545 (Fisher Scientific Co.) were added as carrier. After 30 min the tubes were centrifuged (1000 x g, 15 min) to remove unincorporated [3 H]-TTP. Precipitates were resuspended in 2 ml of a solution containing 1% sodium pyrophosphate and 5% trichloroacetic acid, and collected by filtration through Gelman metrical membranes (0.45 μ pore size) previously layered with 5 mg of Celite. Precipitates were washed with ice cold 5% trichloroacetic acid. They were hydrolyzed in 5% trichloroacetic acid at 80°C for 30 min. Aliquots of the hydrolyzate were added directly to 5 ml Aquasol liquid scintillation

fluid (New England Nuclear) or were neutralized with NH_4OH and mixed with 10 ml Bray's solution¹⁵. Radioactivity was determined in a Unilux II, Nuclear Chicago Liquid scintillation spectrometer with a Ba^{133} external standard. Counting efficiency was 25% in Aquasol and 16% in Bray's solution. All counts were corrected for background and converted to disintegrations per minute (DPM) in order to calculate the pmoles of tritium-labeled thymidine triphosphate ($[\text{}^3\text{H}]\text{-TTP}$) incorporated. Results were expressed as pmoles of $[\text{}^3\text{H}]\text{-TTP}$ incorporated per mg protein or nuclear DNA.

Proteins were extracted from other aliquots of the cytoplasmic fraction by a modified Schneider procedure¹⁶. Protein content was estimated by the procedure of Lowry et al.¹⁷ with bovine serum albumin serving as a standard (Nutritional Biochemical Corp., fraction V).

The nuclear assays were treated similarly except that 6% perchloric acid was used instead of trichloroacetic acid and 95% ethanol and ethanol:ether (1:1) washes of the acid precipitates were included. DNA

concentration was determined by the Burton procedure¹⁸ as modified by Giles and Meyers¹⁹. Salmon sperm DNA (Calbiochem, highly polymerized) was used as a standard.

D. Preparation of DNA

Native DNA was prepared by dissolving calf thymus DNA (Sigma Chemical Co., type I, highly polymerized) in 0.02 M Tris-HCl, 5 mM MgCl₂, (pH 8.5) to a final concentration of 350 µg per ml. A portion of this DNA was denatured by heating at 100°C for 15 min followed by rapid cooling on ice. Another part was "activated"²⁰ by incubation at 16°C for 3 min with 1 µg per ml pancreatic DNAase (Worthington Chemical Co., DPFF, electrophoretically purified from RNAase activity) followed by heating at 90°C for 15 min to destroy enzyme activity.

Results

Cytoplasmic DNA polymerase activity

The cytoplasmic fraction from rat anterior pituitary glands showed maximal DNA polymerase activity in the standard incubation medium in the presence of activated DNA. Incorporation of [^3H]-TTP with the activated DNA was 60 times that of the cytoplasm alone and about four times that of the activity with either native or heat denatured DNA. DNA incubated in the absence of enzyme incorporated negligible amounts of [^3H]-TTP. Maximal activity occurred with all 4 deoxytriphosphates present. Omission of three of them from the standard medium caused activity to decrease by 55%.

The enzyme activity increased linearly with concentration of cytoplasmic protein up to 600 μg (Fig. 1A) and remained linear from 10 to 60 min (Fig. 1B). Under standard conditions, the cytoplasmic fraction from anterior pituitary glands of 38-49 day old animals incorporated 40 pmoles [^3H]-TTP per mg protein in 10 min of incubation.

Changes in activity with age and estrone treatment

Results of in vivo and in vitro studies with rat anterior pituitary glands have shown that changes in DNA synthesis occur during aging^{1,6}. We³ have previously reported a five fold decrease in pituitary DNA synthesis between 46 and 100 days of age. To test if DNA polymerase activity decreased similarly, we assayed the cytoplasmic fraction from anterior pituitary glands of animals ranging from 25 days to over a year in age. We found that the DNA polymerase activity decreased with increasing age (Fig. 2). The activity continued to decrease in animals from 25 days to over a year old (retired breeder rats). The average activity found in cytoplasm from 25 to 32 day old rats, 64 pmoles [³H]-TTP incorporated per mg protein in 10 min, was about four times that of the cytoplasm of the retired breeders. These assays were routinely carried out for 10 and 20 min, times when the kinetics of incorporation were linear for all ages studied.

To test whether the cytoplasm contained factors which stimulated or inhibited the DNA polymerase

activity, equal concentrations of cytoplasmic fractions from 25 day and retired breeder rats were mixed. Aliquots were diluted and assayed for 10 min. The activity of the mixture corresponded exactly to the average of that of the two component cytoplasms (Fig. 3). Thus there appeared to be no specific factors in either cytoplasmic preparation which acted on the enzymes to modify activity.

In addition to the changes brought about by aging, it is known that mitotic activity increases in the anterior pituitary glands of female rats in estrus^{1,9} and in male and female rats injected with estrogen^{5,21}. Administration of estrone (10 μ g on alternate days) to 46-51 day male rats for a week causes a 2 to 3 fold increase in [³H]-thymidine incorporation into intact pituitary glands incubated in vitro²². To measure if DNA polymerase activity was also stimulated by the hormone treatment, cytoplasmic fractions were prepared from pituitaries of estrone treated male rats. The DNA polymerase activity of the estrone treated animals increased to 1.5 times that of the correspond-

ing controls. Assays were carried out under conditions of linear kinetics and with activity proportional to protein concentration.

DNA polymerase activity associated with isolated nuclei

Although DNA polymerase from mammalian tissue can be found in the soluble fraction after centrifugation of a homogenate, nuclei isolated under the proper conditions retain at least part of the DNA polymerizing enzymes²³. It was of interest, therefore, to test DNA polymerase activity in nuclei as well as in the cytoplasmic fraction isolated from anterior pituitary glands of animals of various ages or after estrone treatment.

Nuclei, isolated as described in the methods section, were assayed in the standard incubation medium in the presence of activated calf thymus DNA. The activity with the added DNA was 3.7 times that of the nuclei alone. Native or heat denatured DNA had little effect on the endogenous level of incorporation. When nuclei from pituitary glands of young and old rats were incubated with the activated DNA, the DNA poly-

merase activity of 25 day old animals was twice that of the retired breeder rats (Table I). In these experiments the exogenous template was not rate limiting since three concentrations of nuclei (30, 50 and 70 μ g DNA) from young and old animals showed linear activity with the same amount of added DNA (35 μ g). In similar experiments, nuclei from anterior pituitary glands of estrone treated animals averaged about 13% greater activity than those of corresponding controls (Table I). There was about a 3.5 fold stimulation of activity in the presence of calf thymus DNA compared to the endogenous activity for both nuclear preparations.

Endogenous DNA synthesis in isolated nuclei

Isolated nuclei have been shown to synthesize DNA in the presence of added substrates and co-factors using DNA of the nuclei as template²⁴⁻²⁹. Because our preparations of isolated pituitary nuclei showed DNA synthesizing activity in the absence of exogenous template, we assayed the nuclei to measure if the activity correlated with age and estrone treatment of

the animals.

To measure the nuclear DNA synthesizing activity with endogenous template, the isolated nuclei (40-80 μ g DNA) were incubated in the standard incubation medium without added DNA or cytoplasmic fraction. We found that nuclei prepared from anterior pituitary glands of various ages as well as the estrone treated animals incorporated [3 H]-TTP at the same rate, about 34 pmoles per mg DNA in 10 min.

The incorporation appeared to be due to actual synthesis of new DNA for the following reasons: The [3 H]-TTP incorporation dropped to 25 and 14% of the maximum when 1 or 3 of the deoxyribonucleoside triphosphates were omitted from the reaction mixture. This result suggests that incorporation is not due only to terminal addition of triphosphates. Omission of ATP from the incubation mixture decreased activity to 55% of the maximum. A specific role for ATP in the initiation of DNA synthesis has been suggested³⁰. Third, the incorporated [3 H]-TTP was sensitive to pancreatic DNAase (100 μ g caused release of 96% of the radio-

activity) but not RNAase. Fourth, in a CsCl density gradient sedimentation of DNA extracted from isolated nuclei after incubation, the radioactivity coincided exactly with the pituitary DNA. Finally, radioautographs of nuclei after a 20 min incubation showed grains associated with $3.4 \pm .08\%$ of them. This value agrees well with the 1-2% labeling indices of pituitary incubated in vitro^{3,31}.

In view of reports that DNA synthesis in isolated nuclei can be stimulated by cytoplasmic factors²⁴⁻²⁹, we co-incubated nuclei with various amounts of cytoplasmic fraction. After a 5 min co-incubation there was an average of a 1.9 fold ($187.7 \pm 18.5\%$) stimulation of endogenous activity which was not proportional to the amount of cytoplasmic fraction (50-1000 μ g protein) and did not correlate with the source of the cytoplasmic fraction (young, old, estrone treated animals). With longer periods of incubation the results were variable but showed generally that there was less stimulation and sometimes a depression. After 10, 20 or 30 min the average activity $\pm$ S.E. was 138.3 ± 10.1 , 119.8 ± 13.8 ,

and $91.9 \pm 22.2\%$ respectively, of the endogenous activity in nuclei alone. Similar kinetics reported by Teng et al.²⁸ with L cell nuclei are explained as the rapid breakdown of a labile DNA product made by cytoplasmic DNA polymerase acting on nuclear template. Experiments designed to demonstrate DNAase activity in the cytoplasmic fraction under the conditions of our assay gave negative results.

The results of these co-incubation experiments do however, suggest that the low level of synthesis in the isolated nuclei is not due to lack of DNA polymerase since the cytoplasmic fraction preparations contain about 75% of the gland's DNA polymerase activity. The presence of an excess of nuclear polymerase is also shown in an experiment (Table II) in which nuclei were incubated in standard medium for 10 min, centrifuged and the supernatant withdrawn. The nuclei were resuspended in fresh radioactive medium and incubated for an additional 10 min. Activated DNA was added to the supernatant fraction. This was also incubated for an additional 10 min. In other tubes

nuclei alone or nuclei plus DNA were incubated for 10 min. The calculations of Table II show that calf thymus DNA incubated alone had as much activity as when it was incubated with the nuclei directly (2835 DPM vs 2943 DPM). The exogenous DNA must therefore act with polymerase that leaches from the nuclei into the medium during the incubation. Even after removal of the medium containing these enzymes the nuclei continued to synthesize DNA at the same original rate (3147 DPM vs 3382 DPM). This result strongly suggests that DNA polymerases which can use an activated template are in excess in isolated pituitary nuclei.

Discussion

In many mammalian systems, levels of this soluble DNA polymerase activity have been successfully correlated with cell proliferation during normal development and aging^{32,33,34} as well as hormonally^{35,36}, chemically³⁷,³⁸ or mechanically induced tissue growth^{39,40}.

The present study reveals that total DNA polymerase activity in the anterior pituitary gland of the male rat decreases with the age of the animal and can be increased by in vitro estrogen treatment (Table III). These changes in activity correlate with changes in [³H]-thymidine incorporation into DNA under the same physiological conditions of aging and hormone treatment.

About 75% of the soluble DNA polymerase of the gland was found in the cytoplasmic fraction, a 1000 x g supernatant of a 0.25 M sucrose, 5 mm Mg²⁺ solution, while the remaining 25% was retained by the nuclei. Both polymerase activities reflect the changed rates of DNA synthesis under the various physiological states. The nuclear-associated polymerase however, showed less change than the cytoplasmic enzyme. These data are in

apparent agreement with those of Masui and Garren³⁶ and Coffey et al.³⁵ who report with other tissues that nuclei, when incubated with exogenous DNA, reflect considerably less change in DNA polymerase than do the corresponding cytoplasmic preparations.

The DNA synthesizing activity of the isolated nuclei measured with added calf thymus DNA, was due to soluble polymerase which leached from the nuclei during incubation (Table II). However, the isolated nuclei retain the ability to synthesize DNA in the absence of added template. The incorporation of [³H]-TTP appears to be due to synthesis of new DNA, but the activity did not correlate with the changes in DNA synthesis that occur in the whole gland after manipulation of the animal. Since the nuclei retain excess DNA polymerase, the activity appears to be restricted by the endogenous template. While there are some reports with other systems of a correlation of endogenous activity with that of the DNA synthesizing activity of the intact tissue^{24,27,41} such results are not universal^{25,42-44}. Recently Wallace et al.⁴³

report that nuclei isolated from regenerating liver show a similar lack of template activity for endogenous activity even though the nuclei retain a DNA polymerase detectable by an added DNA.

In view of reports that cytoplasmic control of DNA synthesis can be demonstrated with isolated nuclei^{24,29} we incubated pituitary nuclei in the presence of cytoplasmic fraction. The addition of cytoplasm to nuclei failed to show any specific stimulating or replication control factors. The activity in the co-incubation systems was variable but with 5 or 10 min incubation periods there was an average of 1.6 fold increase in endogenous nuclear activity. This activity is similar to that reported by Lynch et al.²⁷ with regenerating liver nuclei. The measured [³H]-TTP incorporation in such an incubation system is probably the net result of the cytoplasmic content of the deoxyribonucleoside triphosphates, DNA polymerases, phosphatases, nucleases and other degradative enzymes. The kinetics of the response, i.e., an initial stimulation after 5 or 10 min and a depression by 30 min

may reflect a DNAase primed activity similar to that described by Teng et al.²⁸ and by Slater and Loeb⁴⁵.

If the cytoplasm of the 2 or 3% of the pituitary cells which have been stimulated to synthesize DNA do contain specific nuclear regulating factors, as have been shown for HeLa cell cytoplasm²⁴, it is conceivable that their effects may either be overlooked in the presence of degradative enzymes or diluted by the relatively large amount of cytoplasmic material from non-dividing cells.

In conclusion, the results from the present study have shown that concomitant with the changes in DNA synthesis in the gland of the male rat there is a corresponding change in DNA polymerase activity. The activity, found both in the cytoplasm and associated with the nuclei, decreases with increasing age over a relatively long time in the life span of the rat. The activity can be rather quickly increased after a week of estrone injections.

Isolated nuclei are also able to synthesize DNA. The limited endogenous activity in the presence of excess polymerizing enzymes as well as the restricted

response to cytoplasmic fraction suggest that the physical state or availability of the template DNA is important in the control of nuclear replication in the anterior pituitary gland.

In future work it may prove profitable to use tumors of the anterior pituitary gland which not only divide more rapidly than normal pituitary tissue in the initial stages of formation, but also continue to secrete hormones⁴⁶. We also plan to utilize a recently developed cell separation system⁴⁷ to obtain individual classes of anterior pituitary cells, and to use nuclei and cytoplasmic fractions from these various cell types to demonstrate factors which maintain the balance between hormone secretion and the synthesis of DNA.

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Table I. DNA polymerase activity of nuclei isolated from anterior pituitary glands of 25 day old and retired breeder rats and from rats treated with estrone. The latter animals, 46-51 days of age were injected subcutaneously with 10 μ g estrone dissolved in 0.15 ml corn oil on alternate days for 7 days. They were killed on the day after the final injection. Isolated nuclei were incubated in the standard incubation medium in the presence of activated calf thymus DNA. The components of the reaction mixture and the assay procedure as described in the Materials and Methods section. The values are the average of 7 determinations.

Treatment	[³ H]-TTP incorporated in 10 min of incubation (pmoles/mg nuclear DNA $\pm$ S.E.)
Age	
25 day old	112.0 $\pm$ 4.1*
retired breeder	52.3 $\pm$ 2.2*
Estrone treated	116.6 $\pm$ 6.2
Vehicle treated	103.1 $\pm$ 6.2

* Values are significantly different (P < 0.001) as determined by Student's t test.

Table II. DNA polymerase activity of isolated anterior pituitary gland nuclei in the presence and absence of calf thymus DNA. Nuclei were incubated in standard medium for 10 min, placed on ice and centrifuged (1000 x g, 20 min). The supernatant was incubated with activated calf thymus DNA for an additional 10 min (No. 3). Fresh medium containing [³H]-TTP was added to the nuclear pellet and the incubation was continued for 10 min (No. 4). Two controls (No. 1, 2) were incubated for 10 min and stopped.

Assays		[³ H]-TTP incorporated (DPM in 10 min of incubation)
Number	Components	
1	nuclei + DNA	6325
2	nuclei (activity due to added DNA)	3382 2943
3	DNA + medium from a previous nuclear incubation	2835
4	nuclei previously incubated for 10 min + fresh medium for 10 additional min.	3147

Table III. Total DNA polymerase activity of nuclear and cytoplasmic fractions of anterior pituitary glands. Isolated nuclei or cytoplasmic fractions were assayed with activated calf thymus DNA as described in the text. [^3H]-TTP incorporation is calculated as the sum of the activities of equivalent amounts of nuclear and cytoplasmic fractions, and expressed as pmoles incorporated per mg nuclear DNA per 10 min. The values are the average of 4 experiments with the 25 day old and retired breeder rats, and 3 experiments with the estrone treated animals. The estrone treatments is described in the legend to Table I.

Treatment	[³ H]-TTP incorporated (pmoles/mg nuclear DNA ± S.E.)	Ratio Activities A/B
Age		
A 25 day	525 ± 48	2.6
B Retired Breeder	204 ± 34	
Estrone-treated		
A	559 ± 2	1.5
B Control	374 ± 1	

Figure 1A. The dependence of the cytoplasmic DNA polymerase activity on the concentration of cytoplasm. Aliquots (0.10 ml) of various concentrations of cytoplasmic fraction were assayed for 30 min in standard incubation mixture with activated calf thymus DNA. Preparation of the enzyme and the assay procedure are described in the Materials and Methods section.

Figure 1B. The kinetics of the incorporation of [^3H]-TTP by cytoplasmic fraction with activated calf thymus DNA. Cytoplasmic fraction contained 300 μg protein.

Figure 2. The incorporation of [^3H]-TTP by cytoplasmic fractions from anterior pituitary glands of rats of various ages. The number of determinations is given in parentheses. The activity of each group is significantly different from the others ($P < 0.001$) except for the two oldest groups ($P < 0.05$). The error bars represent the standard error of the mean (S.E.).

Figure 3. The incorporation of [^3H]-TTP by cytoplasmic fractions from anterior pituitary glands of 28-day (●) and retired breeder (○) rats, and a mixture of the two (Δ). The incubations were carried out for 10 min with 3 concentrations of cytoplasm, as described in the text.

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FIG. 2