*CHANGES IN NUCLEAR AND CYTOPLASMIC RNA IN REGENERATING MOUSE LIVER**

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The nucleus of a mammalian cell contains most of the cellular DNA and is the site of synthesis of a variety of RNA molecules. Many of these are transferred to the cytoplasm to play various roles in protein synthesis. However, many lines of evidence suggest that this transport is selective, leaving many of the RNA molecules in the cell nucleus. The earlier kinetic studies of Harris[1] are more consistent with some turnover of RNA within the nucleus than with obligatory transfer to the cytoplasm. Analyses of rapidly labeled nuclear RNA in HeLa cells by sedimentation methods reveal much greater amounts of polydisperse RNA than would be expected if it were all precursor to cytoplasmic messenger.[2] Further evidence for nuclear RNA turnover comes from experiments with nucleated avian erythrocytes.[3] Hybridization studies established that certain kinds of RNA molecules present in the nucleus of mouse L-cells are absent in the cytoplasm.[4] Furthermore, the RNA restricted to the cell nucleus is considerably more diverse than cytoplasmic RNA.[4] All of these results suggest a mechanism of control involving transport to the nucleus by which only some of those RNA molecules transcribed reach the cytoplasm to be translated into protein.

Earlier experiments using total liver RNA demonstrated a pattern of changes in the transcription of RNA immediately following partial hepatectomy.[5] Similar experiments using fractionated nuclear RNA synthesized at later stages of regeneration show less-dramatic changes.[6] In view of the magnitude of the differences in the synthesis of total RNA, this system offers itself as a convenient vehicle for comparative studies of nuclear RNA synthesis and its transfer to the cytoplasm. The question may be asked whether the response to partial hepatectomy involves predominantly the synthesis of new cytoplasmic messenger RNA's for the protein synthesis which occurs later or whether concomitant changes in the RNA molecules restricted to the cell nucleus are also evident.

Materials and Methods.—Eight-week-old, uniform, healthy, female Swiss Webster mice were utilized in all experiments. They were housed under controlled conditions and were fed a 20% protein diet ad libitum until 24 hr before experimentation, at which time food was removed. Food was restored after 18 hr of fasting and the animals were decapitated 6 hr later. All partial hepatectomies were performed by one person under light anesthesia by a modification of the method of Higgins and Anderson[7] at 8:00 A.M. after 3 hr of feeding. Control animals from the same group of animals were subjected to a sham operation which involved an identical peritoneal incision.

At $2^1/_2$ hr postoperative, animals received 0.1 ml of physiological saline intravenously, containing 1 mc P^{32} orthophosphate or no radioactive precursor. The animals were decapitated 30 min later.

Preparation of DNA: Nuclei were prepared from whole 16-day embryos or from liver of 8-week-old Swiss white females by a modification of the method described by McCarthy and Hoyer.[8] DNA was prepared by the Marmur[9] method as outlined previously.[5] All DNA samples were centrifuged at 18,000 rpm for 1 hr with 0.01% acid-washed Norit after alcohol precipitation to remove traces of polysaccharides and protein. The criteria for clean DNA were spectral proper-

ties; the absence of detectable protein, polysaccharides, or RNA; and the ability to be retained on a membrane filter *and* to remain on the filter for the duration of hybridization reaction.

Preparation of RNA: Total RNA was isolated as described previously[5] from freshly excised, rapidly cooled livers. Cytoplasmic and nuclear fractions were prepared by a modification of the method described by Blabel and Potter.[10] Livers were excised quickly, trimmed, and chilled in 10 vol of ice-cold 0.32 *M* sucrose containing 0.03 *M* $MgCl_2$ and 0.03 *M* tris-HCl, pH 7. After livers were minced with scissors, the sucrose solution was filtered off and an additional 5 vol of the same ice-cold sucrose solution added. Initial homogenization was accomplished by one stroke in a Potter-Elvehjem homogenizer with a motor-driven pestle having 0.020-cm clearance at 2100 rpm. The homogenate was twice filtered through four layers of cheesecloth and the mixture diluted to 0.25 *M* sucrose, *layered* onto the 0.32 *M* sucrose solution, and spun for 5 min at 2500 rpm. The supernatant provided cytoplasm free of nuclei as determined by microscopic examination and was used for extraction of cytoplasmic RNA.

The pellet was carefully taken up in the 0.32 *M* sucrose solution and homogenized in the same homogenizer for an additional six to ten strokes. After centrifugation at 2500 rpm as before, the pellet was taken up in 0.88 *M* sucrose solution and layered onto 10 ml of 2.4 *M* sucrose containing 0.3 *M* $MgCl_2$ and 0.03 *M* tris-HCl. The interphase was stirred and the nuclei were pelleted by centrifugation for 25 min at 40,000 rpm in an International B60 centrifuge with an A112 fixed-angle head. The supernatant was poured off, the cellular material adhering to the tube wall carefully removed, and the tube was wiped clean with tissue paper.[10] The clear white nuclear pellet was taken up in SSC and nuclear RNA extracted with an equal volume of 0.28 *M* LiCl solution as described previously.[5] After lysis of the nuclei, the viscous solution was sonicated in a Branson LS 75 Sonifier for 60 sec at peak output to degrade the DNA. An equal volume of 65°C phenol was added and the mixture shaken and allowed to remain at 65°C for 5 min before cooling and centrifugation. The supernatant was precipitated overnight at −20°C with 2 vol of ethanol, digested with DNase, treated with phenol, and after precipitation passed through a G50 Sephadex column.

Hybridization of RNA with DNA: DNA dissolved in 0.1 × SSC at 100 μg/ml was heat-denatured at 95°C for 10 min and quickly cooled in a dry-ice acetone bath in 1000 ml of 4 × SSC. DNA was immobilized on membrane filters[11] by passing a 10 μg/ml DNA solution through 145-mm filters. Filters were cut to the required size and DNA content was checked by the diphenylamine reaction[12] for uniformity of distribution.

The hybridization reaction between 15 μg pulse-labeled RNA and 15 μg DNA immobilized on 5-mm-diameter filters was carried out in 0.8 ml of 4 × SSC at 67°C for 16 hr. The filters were then removed from the reaction vials, washed three times in 4 × SSC at 67°C, dried, and counted in a Packard Tri-Carb scintillation counter.

In competition experiments, the desired mixtures of pulse-labeled and unlabeled RNA in 4 × SSC were mixed and heated to 67°C before addition of the DNA filter. Inputs were calculated from 10% TCA-precipitable RNA counts of parallel RNA mixtures carried through the reaction conditions without DNA filters. *B. subtilis* DNA filters were used in an exactly parallel series to determine heterologous background for each point. The background hybridization was always less than 0.05% of the input counts.

Results.—A clear separation between nuclear and cytoplasmic RNA can only be achieved when homogenization is minimal. Table 1 shows the results of varying the number of strokes of the homogenizer used for pulse-labeled normal liver. As the number of strokes is increased, the difference between the apparent specific activities of nuclear and cytoplasmic RNA diminishes, presumably resulting from the breakage of some of the more fragile nuclei. Since the tissue contains more than one type of cell, a considerable variation in the fragility of both cells and nuclei is to be expected. With only a single homogenizing stroke, the specific activity ratio was fairly consistent between different runs. However, in view of this fragility it is clear that some contamination of cytoplasm by nuclear material is inevitable and differences observed between the two RNA preparations will be minimal estimates of the real difference.

TABLE 1

SPECIFIC RADIOACTIVITY OF RNA FRACTIONS OBTAINED BY VARYING DEGREES OF HOMOGENIZATION

No. of homogenizer strokes	Specific radioactivity of nuclear RNA / Specific radioactivity of cytoplasmic RNA
1	20.1, 23.2, 17.6
4	9.1, 13.9, 11.8
8	4.3, 2.8, 3.2

The appearance of P^{32} in nuclear and cytoplasmic liver RNA after various periods following injection of 8-week-old females with labeled orthophosphate is shown in Figure 1. The specific radioactivity of nuclear RNA rises very quickly after the intravenous injection, to reach a maximum by about 30 minutes. During this period there is a very low level of labeling in the cytoplasm. The specific radioactivity of cytoplasmic RNA rises steadily, to approach that of nuclear RNA by 24 hours. While this labeling pattern is evidence for a precursor product relationship between nuclear and cytoplasmic RNA it cannot exclude some possible turnover of RNA within the nucleus.

Very similar labeling data was obtained from experiments with regenerating liver. In both normal and regenerating liver, the difference in the specific activity between nuclear and cytoplasmic RNA for pulses of 30 minutes was at least a factor of 15. This difference was routinely used as a means of monitoring the purity of cytoplasmic RNA preparations. Unlabeled RNA was prepared in parallel with each labeled preparation so that the distribution of radioactivity between the two fractions of the latter could be used as a criterion of purity.

DNA/RNA hybridization reactions using pulse-labeled nuclear RNA from normal liver and cytoplasmic RNA prepared after various degrees of homogenization are illustrated in Figure 2. Cytoplasmic RNA prepared with one stroke of the homogenizer competed for only a part of the labeled nuclear RNA. Cytoplasmic RNA contaminated with nuclear RNA due to breakage of fragile nuclei was a better competitor, and when ten strokes of the homogenizer are employed, no plateau is achieved. It may therefore be concluded that there are some labeled RNA molecules in the nucleus which are very rare or essentially absent in the cytoplasm. Similar results were obtained previously with mouse L-cells and rabbit kidney cells.[4] This unique nuclear RNA appears to amount to some one third of the hybridizing nuclear RNA, although this must be a minimal estimate since some nuclear contamination of the cytoplasmic RNA may still occur.

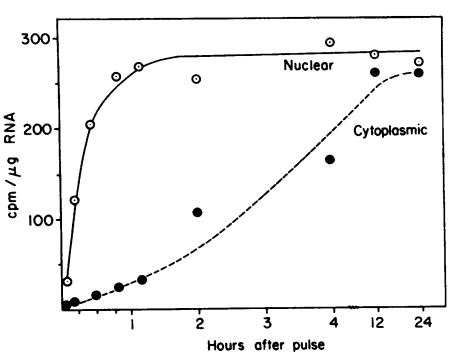

FIG. 1.—Rate of labeling of mouse liver nuclear and cytoplasmic RNA. Eight-week-old Swiss white female mice were injected intravenously with 1 mc of neutralized P^{32} orthophosphate and decapitated at various times thereafter. The incorporation of the isotope into the nuclear and cytoplasmic RNA of the liver is shown as the specific activity of each fraction with time. Each point represents the average of three animals.

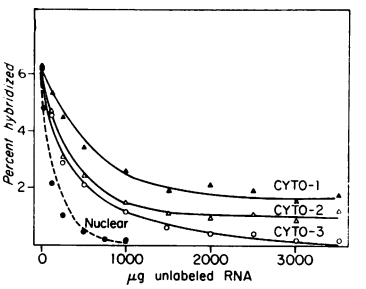

FIG. 2.—Competition of unlabeled RNA from nuclear and various cytoplasmic preparations in the hybridization of P^{32} pulse-labeled RNA from purified normal mouse liver nuclei. Fifteen μg of 30-min P^{32} pulse-labeled RNA was incubated with 15 μg of DNA in 2 ml of 4 × SSC for 16 hr at 67°C in the presence of increasing amounts of unlabeled RNA isolated from purified nuclei (*Nuclear*); cytoplasm prepared by one-stroke homogenization (*CYTO-1*); cytoplasm prepared by five homogenization strokes (*CYTO-2*); and cytoplasm prepared by ten homogenization strokes (*CYTO-3*).

Comparisons among the RNA molecules present in nucleus and cytoplasm of normal liver with the corresponding regenerating liver fractions are illustrated in Figure 3. This experiment and all others to be described subsequently utilized cytoplasmic RNA fractions obtained from single-stroke homogenates. The top figure indicates that nuclear RNA was able to compete for both preparations of cytoplasmic RNA more efficiently than cytoplasmic RNA. Thus, a greater concentration of the RNA species which hybridize exists in the nucleus as compared to the cytoplasm. Both regenerating nuclear and cytoplasmic competitor RNA (not shown) compete fully with normal cytoplasmic rapidly labeled RNA. This result is in accordance with our previous finding that all of the hybridizable RNA present in normal liver can be competed for by similar molecules in regenerating liver.[5]

In the lower portion of Figure 3 are shown competition curves of normal and regenerating RNA in the hybridization reaction of pulse-labeled normal nuclear RNA with filter-bound DNA. Normal cytoplasmic RNA was unable to compete against a substantial portion of the RNA sequences represented in nuclear RNA, as indicated by the high plateau value. Regenerating cytoplasmic RNA, on the other hand, is a much more effective competitor. This implies that some of the RNA sequences which normally are *unique* to the nucleus are now present in the cytoplasm although the slope of the competition curve suggests that these molecules are not as prevalent as in the normal nucleus. Both normal and regenerating nuclear competitor RNA compete very efficiently against rapidly labeled normal nuclear RNA, in agreement with evidence for total pulse-labeled RNA.[5]

Rapidly labeled nuclear and cytoplasmic RNA from three-hour regenerating mouse liver was used in the competitive hybridization reactions shown in Figure 4. In agreement with the evidence presented previously for total RNA,[5] the RNA species represented in the normal spectrum do not compete fully with regenerating RNA. In the earlier work, the competition curves were not carried out to high levels of competitor due to the solubility restrictions imposed by the limited reaction volume. Normal cytoplasmic RNA is the least efficient competitor and normal nuclear RNA does not appear to contain all of the sequences present in the rapidly labeled regenerating cytoplasmic RNA. At low levels of normal nuclear RNA, the competition is quite effective as compared to homologous regenerating cytoplasmic RNA. Thus, the more prevalent molecules present in the cytoplasmic fraction of regenerating liver seem to be present in normal liver but only in the nuclear fraction.

The lower portion of Figure 4 demonstrates that there are molecules present in

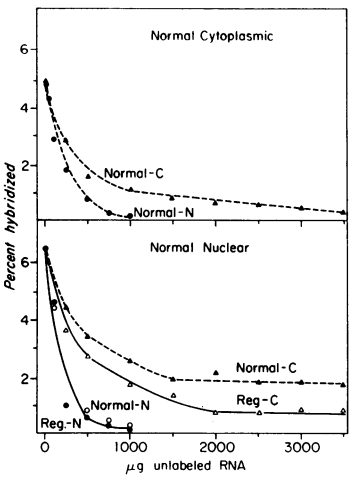

FIG. 3.—Competition between nuclear and cytoplasmic RNA in the hybridization reaction of pulse-labeled cytoplasmic RNA (*top*) and nuclear (*bottom*) isolated from mouse liver. Hybridization conditions as described in Fig. 2. Unlabeled RNA isolated from normal nuclei (*Normal-N*), normal cytoplasm (*Normal-C*), regenerating cytoplasm (*Reg-C*). Regenerating liver nuclei and cytoplasm isolated 3 hr after partial hepatectomy.

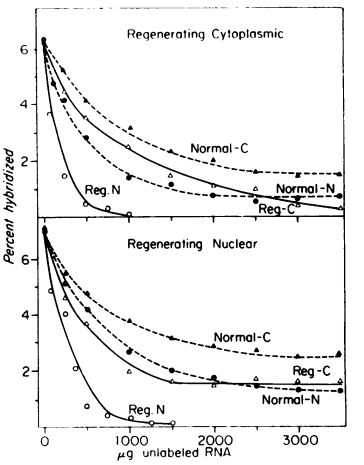

FIG. 4.—Competition of unlabeled RNA from normal and regenerating liver cell fractions, as defined in Fig. 3, in the hybridization of pulse-labeled regenerating cytoplasmic RNA (*top*) and nuclear RNA (*bottom*) in the reaction with membrane-bound DNA under the conditions outlined in Fig. 2.

regenerating liver nuclei which are absent in the cytoplasm as in the case of normal liver and other cells. Since normal nuclear RNA is a more efficient competitor than normal cytoplasm, some of these unique nuclear RNA molecules are also restricted to the nucleus in normal liver. This conclusion is supported by a more direct experiment involving additive competition. Various amounts of cytoplasmic RNA from regenerating liver were added to regenerating nuclear RNA to establish the plateau representing hybridized RNA unique to the nucleus. As shown in Figure 5, this is achieved with approximately 2 mg of cytoplasmic RNA. The rest of the curve is established by mixing various quantities of normal or regenerating nuclear RNA each with 2 mg of regenerating cytoplasmic RNA. As would be expected, regenerating nuclear RNA is an effective competitor while normal nuclear RNA is only partially effective. Thus, of the RNA molecules restricted to the nucleus in regenerating liver some are also present in normal liver nuclei while others are characteristic of the regenerating tissue.

Discussion.—The populations of RNA molecules in normal and regenerating liver were analyzed and compared in the present work and in previously published studies[5] by DNA/RNA hybridization methods. In view of recent investigations of the nature of hybridization reactions in vertebrate systems, it is perhaps appropriate to first discuss the interpretation and limitations of this assay.

It has been clear for some time that the formation of DNA/RNA hybrids does not demand absolute complementarity of base sequence by the two participating

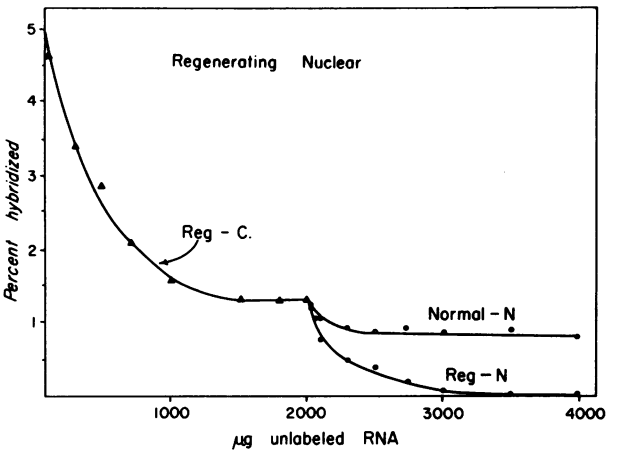

FIG. 5.—Competition of unlabeled RNA from regenerating cytoplasm in the reaction of 15 μg of P^{32} pulse-labeled regenerating nuclear RNA with 5 μg of DNA to a total of 2 mg of this unlabeled RNA. The extended competition of unlabeled normal and regenerating nuclear RNA in the second part of the reaction involves addition of up to 2 mg of these nuclear preparations to 2 mg of unlabeled regenerating cytoplasmic RNA to reach a cumulative total of 4 mg of competitor RNA.

strands. This is evident from the cross reactions obtained between RNA and DNA originating from two related species and from the competitive effect of RNA from a related species on a homologous reaction.[13] Recent published studies on DNA/DNA duplex formation reactions with mammalian DNA demonstrate that these reactions do not have complete locus specificity.[14-16] This results from the fact that there are many similar but not identical nucleotide sequences within a given mammalian DNA.[16] The existence of such intragenome homologies allows the formation of duplex structures in which the two participating DNA strands originate from different genetic loci. Such structures may often be recognized by their low thermal stability as compared to native or *bona fide* renatured DNA.[14, 15] Since there are many base sequences which are interrelated to a greater or less extent, the precise nature of these mispaired structures is highly dependent upon the reaction conditions.[15]

Similar considerations apply to DNA/RNA hybrid formation[17] so that RNA molecules react with the DNA of genetic loci other than those responsible for their synthesis. Again, the extent of hybridization and the extent of competition are highly dependent upon the reaction conditions since these govern the occurrence of reactions between the RNA and DNA of similar, but different, genetic loci.[18] Thus, at low salt concentrations or high temperatures, only the most highly paired structures will be stable.[15] Therefore, the hybridization assay with mammalian nucleic acids is better viewed as a chromatographic system in which there are a great number of different adsorption sites than one in which specific cistrons are titrated with their own gene product. In view of the existence of families of similar base sequences, the specificity of the adsorption is limited. Thus, two RNA molecules synthesized at similar, but not identical, sites may well be indistinguishable. Therefore, in competition experiments, observed differences must reflect real differences although failure to discriminate does not prove identity. In fact, differences may be demonstrated in these latter cases by the use of more discriminating reaction conditions,[18] as in the case of interspecific DNA/DNA reactions.[15]

These considerations apply only to a portion of mammalian DNA. Not all regions of the DNA have such base sequence relationships with other areas of the genome.[16] Since RNA molecules produced at these sites have no opportunity for

these less-specific reactions, their relative rate of reaction is very much lower and the assay would tend to select against them. With these limitations in mind, the interpretation of the present results will be discussed.

A major fraction of the hybridizable RNA of normal liver nuclei is absent or present only in very small amounts in the cytoplasm. This result is entirely analogous to that obtained in mouse L-cells and rabbit kidney cells.[4] The same situation occurs in the cells of regenerating liver. The primary concern of the present experiments was with the relative changes in the nuclear and cytoplasmic RNA of regenerating liver as compared to the normal organ. Changes in cytoplasmic RNA are certainly to be expected as a corollary of increased protein synthesis during regeneration. This effect is observed and accounts for some of the new species of RNA synthesized in response to partial hepatectomy. In addition, however, it is clear that many of the new species of RNA in regenerating liver are restricted to the nucleus. Thus, the increased transcription is not completely dedicated to the production of new cytoplasmic messages.

The nature and function of the RNA molecules which are restricted to the nucleus is still obscure. Conceivably, their function is connected with the synthesis of nuclear proteins and the mitotic apparatus. Alternatively, this fraction of RNA may be potentially cytoplasmic messenger RNA but is turned over within the nucleus if the mechanisms transporting it to the cytoplasm fail. Since the hybridization experiments demonstrate that unique nuclear RNA molecules are qualitatively different from those transferred to the cytoplasm, this proposition demands that the transportation process be selective. Thus, in addition to control at the level of transcription, a further selection of those molecules to be translated could be made at the nuclear membrane or earlier during the attachment of ribosomes.

The present data offer some support for these ideas by virtue of the results presented in Figure 3. These imply that some RNA molecules present only in the nucleus in normal liver appear in the cytoplasm in regenerating liver. This could be a result of an alteration in the selective transportation system. However, this result is not entirely conclusive since, as discussed earlier, the less than absolute specificity of the hybridization reactions allows competition by similar, but not necessarily identical, RNA molecules. Thus, the result may be attributed to the existence of cytoplasmic RNA molecules in regenerating liver very similar in base sequence to nuclear RNA molecules in normal liver nuclei. Although this would seem an unlikely explanation, it cannot be eliminated at the moment.

Summary.—Both normal and regenerating mouse liver contain some RNA molecules restricted to the nucleus and essentially absent from the cytoplasm. The changes which occur in RNA synthesis in the liver following partial hepatectomy result in changes in the RNA of both nucleus and cytoplasm. Some of these new species of RNA are restricted to the nucleus. Some RNA molecules restricted to the nucleus in normal liver appear in the cytoplasm of regenerating liver. It is suggested that selective transport of RNA to the cytoplasm may be an important device for the regulation of translation of potential messengers.

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[1] Harris, H., in *Progress in Nucleic Acid Research*, ed. F. N. Davidson and W. E. Cohn (New York: Academic Press, 1963), vol. 2, p. 19.

[2] Penman, S., *J. Mol. Biol.*, **17**, 117 (1966).

[3] Attardi, G., H. Parnas, M. I. H. Hwang, and B. Attardi, *J. Mol. Biol.*, **20**, 145 (1966); Scherrer, K., and L. Marcaud, *Bull. Soc. Chimie, Biol.*, **47**, 1697 (1965).

[4] Shearer, R. W., and B. J. McCarthy, *Biochemistry*, **6**, 283 (1967).

[5] Church, R. B., and B. J. McCarthy, *J. Mol. Biol.*, **23**, 459 (1967); *ibid.*, p. 477.

[6] Drews, J., and G. Brawerman, *J. Biol. Chem.*, **242**, 807 (1967).

[7] Higgins, G. M., and R. M. Anderson, *A. M. A. Arch. Pathol.*, **12**, 186 (1931).

[8] McCarthy, B. J., and B. H. Hoyer, these PROCEEDINGS, **52**, 915 (1964).

[9] Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961).

[10] Blabel, G., and V. R. Potter, *Science*, **154**, 1662 (1966).

[11] Gillespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965).

[12] Burton, K., *Biochem. J.*, **62**, 315 (1956).

[13] McCarthy, B. J., and E. T. Bolton, these PROCEEDINGS, **50**, 156 (1963).

[14] Walker, P. M. B., and A. McLaren, *J. Mol. Biol.*, **12**, 394 (1965).

[15] Martin, M. A., and B. H. Hoyer, *Biochemistry*, **5**, 2706 (1966).

[16] Britten, R. J., and D. E. Kohne, in *Carnegie Institution Yearbook 65* (Washington, D. C.: 1967), p. 78.

[17] Denis, H., *J. Mol. Biol.*, **22**, 269 (1966).

[18] Church, R. B., and B. J. McCarthy, in preparation.