

DNA Replication in Mammalian Cells, I. The Size of Newly Synthesized Helices

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Abstract. The newly synthesized DNA of heteroploid human cells cultured *in vitro* has a single-stranded molecular weight of approximately 1×10^6 following 30- to 60-second pulses of labeling with tritiated thymidine. With increasing pulse times the amount of radioactive DNA having a low single-stranded molecular weight remains approximately constant, whereas increasing amounts of label are found associated with the bulk DNA. After removing the label from the medium, the radioactivity associated with the small molecular weight DNA is chased into high molecular weight material. These findings are analogous to what is observed in microorganisms and suggest a basically common organization of the growing point of DNA in bacterial and in mammalian cells.

Introduction. The work of Okazaki and his collaborators¹ has shown that the newly synthesized DNA of bacteria and T-even phages is extracted in the form of a low molecular weight material, as judged by alkaline sucrose gradient centrifugation. Mitra *et al.*² had proposed in the past that, at least on one of the sister helices, DNA would be synthesized in small pieces, later to be joined by a ligase activity. Several authors³⁻⁷ have shown that polynucleotide ligase seems indeed responsible for binding together *in vivo* the little pieces mentioned above. Small molecular weight DNA seems therefore to be an obligatory intermediate in the replication of the chromosome of microorganisms.

We wanted to establish whether a similar process is taking place also in mammalian cells. In this paper we report that after ³H-thymidine pulses of 30 to 60 seconds, the radioactive DNA of human cells cultured *in vitro* is found to have a sedimentation coefficient in alkaline sucrose gradients of approximately 5S, similar to that observed after a few second pulse in the case of bacterial or phage DNA synthesis and markedly lower than that of the bulk of the DNA. This low molecular weight material can be chased into the bulk DNA. Similar results have been reported by Taylor in Chinese hamster cells,⁸ and more recently by Painter and Schaefer⁹ in HeLa cells.

Materials and Methods. Cell cultures: The experiments have been carried out on a human heteroploid cell line, the EUE,¹⁰ grown in suspension or in monolayer cultures, and on a human diploid cell strain derived from a skin biopsy and maintained *in vitro* for 3 to 4 passages (30 days). The stock suspension cultures of EUE were grown in 300-ml bottles with a floating magnetic stirrer. The medium was Eagle's basal medium for suspension culture¹¹ supplemented with 10% of calf serum, 3 gm/l of lactalbumin, 0.2

gm/l of yeast extract, and 10.5 mg/l of L-serine. The monolayer cultures of both the diploid strain and the heteroploid EUE line were grown in Eagle's medium with double concentration of amino acids and vitamins, supplemented with 10% calf serum. In all the experiments the cells were grown at 37°; in these conditions the generation time is about 20 hr for EUE cells, and 24 hr for the diploid strain.

Reagents: Methyl-³H-thymidine (19 Ci/mole) was purchased from the New England Nuclear Corp. and sodium azide from E. Merck AG, Darmstadt, Germany. Tritiated DNA from *Bacillus subtilis*, degraded with pancreatic DNase to a sedimentation coefficient in alkali of 5S, and sonicated cold *B. subtilis* DNA having a sedimentation coefficient in alkali of 10S, were kindly supplied by Dr. S. Spadari.

Pulse-labeling experiments: ³H-thymidine was added to exponentially growing cells (5 to 7 × 10⁶ cells/ml) in suspension cultures at a concentration of 10⁻⁶ M. At various time intervals, the pulse was terminated by pouring 15-ml samples into 3 vol of ice-cold BSS (Hanks' Balanced salt solution)¹² containing 50 μg/ml of sodium azide. The cells were collected by centrifugation at 0° and washed twice with ice-cold BSS.

Extraction of denatured DNA: The pellet was suspended in 1.5 ml of ice-cold 0.2 N NaOH containing 0.01 M EDTA. The suspension was frozen in liquid nitrogen; each sample was thawed before using and incubated at 37° for 10 min. An aliquot of 0.5 ml was used to assay the total radioactivity incorporated in acid-insoluble material.

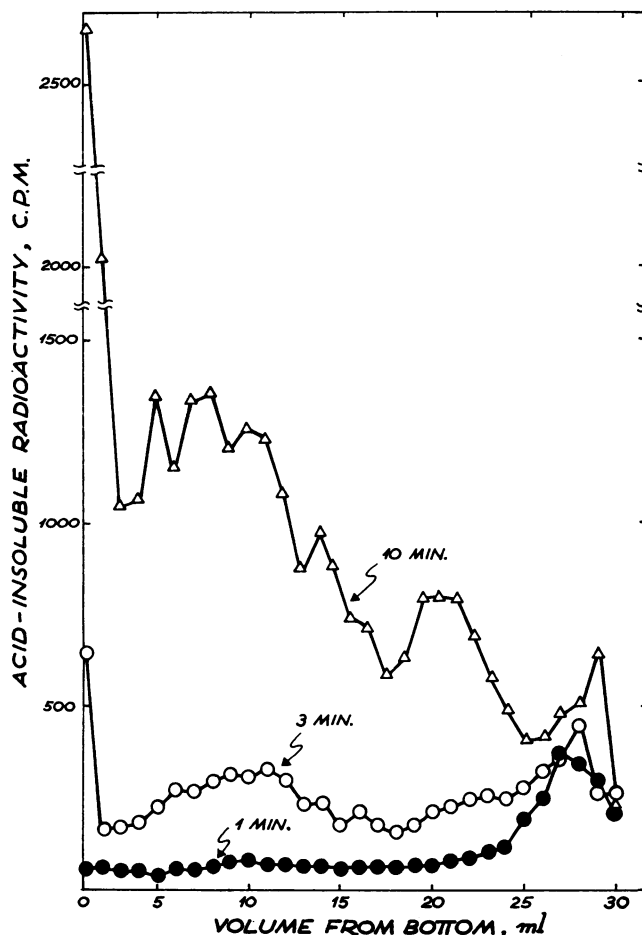
Pulse-chase experiments were performed in monolayer cultures; 10⁻⁶ M ³H-thymidine was added to bottles containing 10 ml of medium and about 3 × 10⁶ exponentially growing cells; after the appropriate time, the medium was removed, the cells were washed twice with pre-warmed medium and, finally, medium supplemented with 10⁻⁶ M cold thymidine was added to the monolayer. The incubation was continued for the desired times, the medium was then poured out, the incubation stopped by the addition of 1.5 ml of 0.2 N NaOH and 0.01 M EDTA, and the suspension was digested at 37° for 20 min; 0.5 ml was used for the determination of the total radioactivity incorporated.

Alkaline sucrose gradients: One milliliter of alkali-digested cells was layered on a 5 to 20% sucrose gradient (29 ml) containing 0.1 N NaOH and 0.9 M NaCl, and centrifuged at 0° in rotor SW 25 of the Spinco centrifuge, model L, for 13 hr at 22,500 rpm. One-milliliter fractions were collected from the bottom of the tube, precipitated with ice-cold 2 N HCl, collected onto glass filters (Whatman GF/C, 2.5 cm diameter), washed, dried, and counted in a Packard Liquid Scintillation Counter.

Results. Pulse labeling of DNA: The results of two typical experiments are reported in Figures 1 and 2. Alkaline digests of EUE cells, pulse-labeled for different times (35 sec to 43 min), were centrifuged in alkaline sucrose gradients, and the acid-insoluble radioactivity in each region of the gradient was measured. After pulses of 35 seconds or 1 minute, the sedimentation pattern of the ³H-DNA shows a well-defined peak only at the top of the gradient where most of the radioactivity is found. With increasing pulse times (from 3 to 10 min) the slowly sedimenting material can still be seen, while increasing values of radioactivity are found in the fast-sedimenting zones. At longer pulse times, it was impossible to distinguish the small material from the tail of the bulk of the DNA. Identical results were obtained by pulse-labeling for 1, 5, and 20 minutes human diploid fibroblasts from primary monolayer cultures.

Chase of the radioactive DNA into high molecular weight material: The data of Figures 1 and 2 indicate that the newly synthesized DNA is present as small fragments with a sedimentation coefficient definitely different from that of the bulk DNA. The relatively constant level of the radioactivity in the slow sedimentation zone and the increasing levels in the faster one, after longer incubation times with ³H-thymidine, suggest that the small DNA fragments are

FIG. 1.—Sedimentation profiles in alkali of pulse-labeled DNA from EUE cells. H^3 -thymidine was added to a suspension culture in logarithmic phase containing 5.7×10^5 cells/ml; 15-ml samples of the suspension were removed at the indicated times; the growth was stopped, and the cells were washed, concentrated, and digested in alkali; they were then analyzed for the size of the radioactive DNA in an alkaline sucrose gradient. For the procedure, see *Material and Methods*.



precursors of the higher molecular weight DNA. On the basis of this hypothesis we would expect that a chase with cold thymidine should lead to the disappearance of the radioactivity from the top of the gradient. Such an experiment is better performed in monolayer cultures where the radioactive thymidine is easily and quickly replaced by the cold one; Figure 3 shows that incorporation of radioactive precursors is indeed blocked by this procedure. The size of the radioactive DNA as studied with alkaline sucrose gradients can be seen in Figure 4. The small radioactive DNA molecules appearing in the sedimentation profiles of DNA pulse-labeled for 30 seconds, 1, 3, and 5 minutes are no longer present in the gradients of cells extracted after the chase, where all the radioactive DNA has a high sedimentation coefficient.

Sedimentation coefficient of the small DNA: The S value of the newly formed DNA was estimated with DNA of known molecular weight as reference. In Figure 5 are shown the sedimentation patterns in alkaline sucrose gradient of DNA from EUE cells pulse-labeled for 1 minute, that of a DNase-treated 3H -DNA from *B. subtilis*, having an average sedimentation coefficient of $5S$

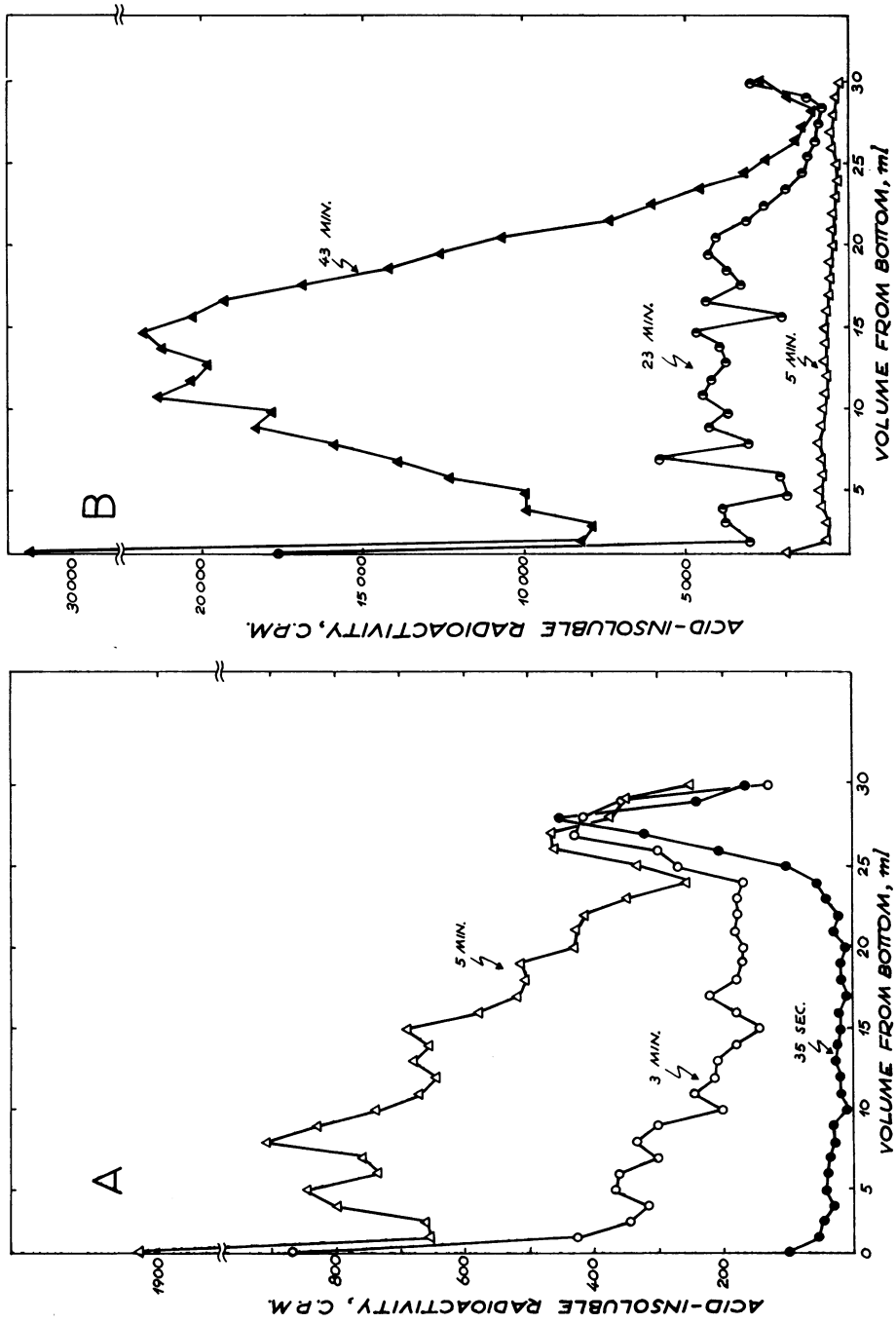


Fig. 2.—(A and B) Sedimentation profiles in alkali of pulse-labeled DNA. The experiment was similar to that of Fig. 1, except for different pulse times.

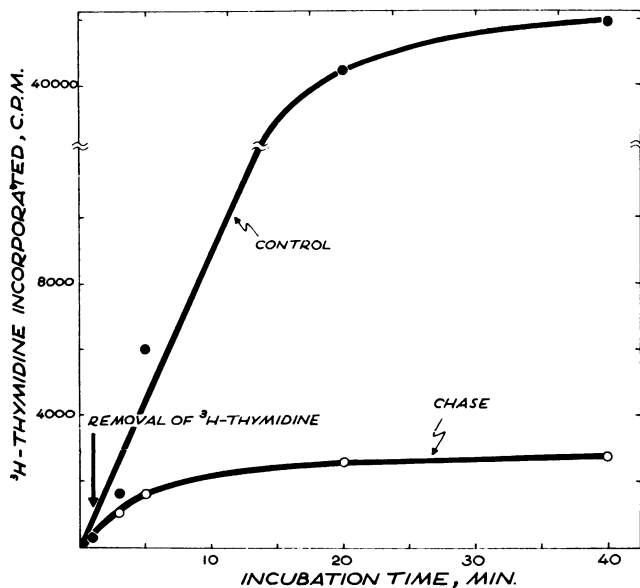


FIG. 3.—Incorporation of ^3H -thymidine into DNA of EUE cells growing in monolayer cultures. Each point corresponds to one bottle containing 3×10^6 cells and 10 ml of medium. 10^{-6} M ^3H -thymidine was added at zero time. In the appropriate bottles the medium was removed 1 min after the addition of ^3H -thymidine; the cells were washed twice, and pre-warmed medium containing 10^{-6} M cold thymidine was added. The cells were collected from the bottle at the indicated times with 1.5 ml of 0.2 N NaOH and 0.01 M EDTA. The suspension was digested for 20 min at 37° and the acid-insoluble radioactivity was assayed on 0.5 ml of the digest. The residual 1 ml was used for the alkaline sucrose gradients reported in Fig. 4 B.

and that of cold DNase-treated *B. subtilis* DNA, having a sedimentation coefficient of 10S, as determined by analytical centrifugation in alkali in both cases. The near coincidence of the two peaks of EUE cells pulse-labeled DNA and of the 5S DNA indicates similar sedimentation coefficients, and therefore the molecular weight of newly synthesized DNA molecules can be estimated of the order of 1×10^6 .

Secondary structure of pulse-labeled DNA: In order to determine whether the new chains were attached with the usual base-pairing bonds to the DNA template, we isolated DNA from the EUE cells following 30- to 60-second pulses of ^3H -thymidine by a variety of techniques like the ones described by Thomas *et al.*¹⁴ and by Yudelevich *et al.*⁴ or variations thereof. The material so extracted was analyzed in neutral sucrose gradients; in all cases the radioactive DNA was found in the top of the gradient. On the other hand, the procedures employed have given evidence of causing extensive degradation of the DNA as judged by the sedimentation coefficient, in alkali or neutral, of the bulk DNA (i.e., of the radioactive DNA after pulses of 5 min or longer) probably because of nuclease action during the extraction. When more drastic extracting conditions were employed such as incubation at 50° for five hours, in 0.25% sodium lauryl sulfate,⁸ there was less evidence of degradation, but the one-minute pulse-labeled

DNA was still found at the top of the neutral gradients. Such conditions may be sufficient to denature the short pieces and detach them from the template DNA. Similar difficulties were found also in the bacterial system by Sugimoto *et al.*³ and by Oishi;¹⁵ variations of the extraction procedure were sufficient, in those systems, to show that most, if not all, of the radioactivity was indeed attached with weak bonds to the bulk of the DNA, as reported by Yudelevich *et al.*⁴ The same procedure of extraction described by the latter authors, when

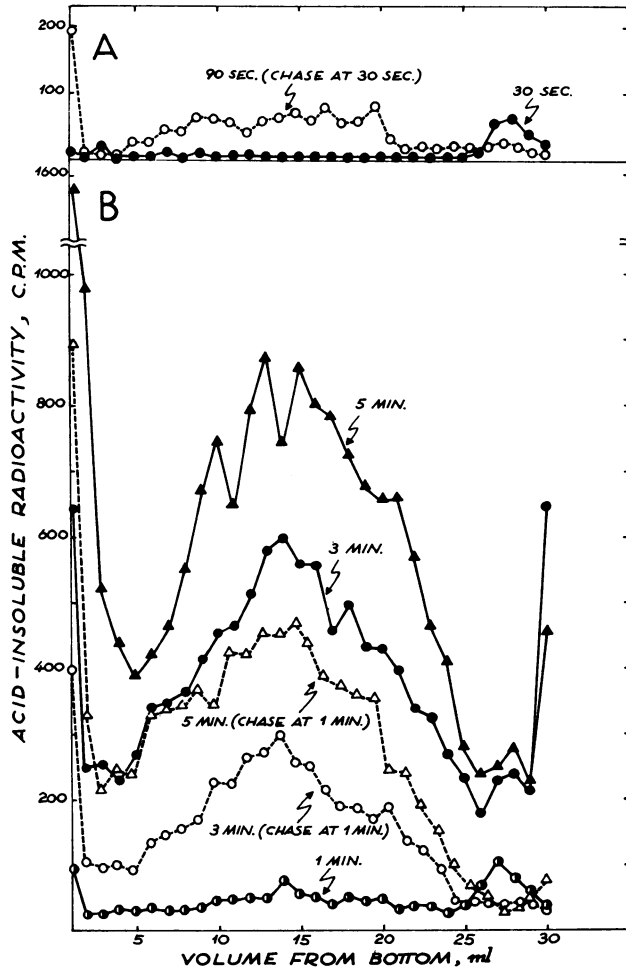
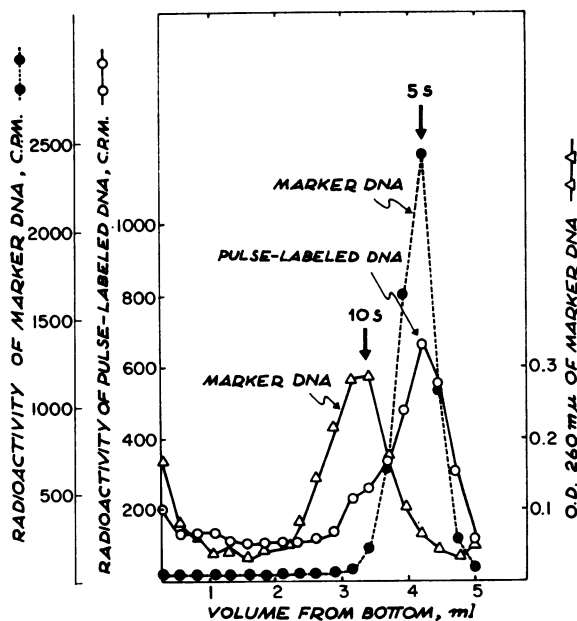


FIG. 4.—(A and B) Sedimentation profiles in alkali of DNA following pulse-labeling and chase. The cells were grown in monolayer and the pulse and chase procedure was performed as in Fig. 3. The solid graphs indicate the pulse experiments, and the dotted ones indicate the corresponding chases. In Fig. 4A the chase was performed 30 sec after the addition of ^3H -thymidine and the incubation continued for another 60 sec. In Fig. 4B the chase was performed in the appropriate bottles after 1 min, and the incubation continued for the indicated times. Each gradient corresponds to one bottle and to one point of the graphs of Fig. 3, where the total radioactivity incorporated at each time is reported.

FIG. 5.—Sedimentation profiles in alkaline sucrose gradients of single-stranded ^3H -DNA from *B. subtilis* degraded with pancreatic DNase, of sonicated cold *B. subtilis* DNA, and of ^3H -DNA from EUE cells. Bacterial DNA's were run in the same tube, and EUE cells DNA in a parallel tube of the SW50 rotor of the Spinco Ultra-centrifuge for 5 hrs at 47,000 rpm, at 0° . The sedimentation coefficient of the bacterial DNA's had been determined by analytical centrifugation in alkali according to the technique of Studier.¹³



applied to the EUE cells, did instead yield a markedly degraded DNA, as judged by the behavior in neutral or alkaline gradients of the bulk material. Conceivably the more sheltered situation of the DNA in mammalian cells, compared to the bacterial systems, allows more time for nucleases to act on it during the relatively mild extracting conditions employed. When more violent procedures are used, they are probably sufficient to denature the DNA at the growing point, whereof many authors have described the pronounced instability.¹⁶

Estimate of the number of pieces per growing point: The time taken for the synthesis of each of the 300 nucleotide-long pieces is of the order of one minute as judged by our results; the cultures were not treated in any way to slow their doubling time (of approximately 20 hr at 37°). Huberman and Riggs¹⁷ estimated that in HeLa (a strain in many ways similar to EUE, and having a comparable division time) the rate of movement of the growing point is between 1.5×10^3 and 8×10^3 nucleotides per minute per single chain. If we can apply these figures to our cells, we can estimate between 5 to 25 pieces per single strand at any growing point (10 to 50 pieces per growing point).

Discussion. The results reported above indicate that polydeoxynucleotides having a single-stranded molecular weight of about 1×10^5 are intermediates in the synthesis of human cell DNA. This does not seem to be a peculiar property of a particular heteroplloid strain, since diploid cultures also exhibit the same pattern; a similar conclusion was drawn from studies made in Chinese hamster culture by Taylor⁸ and in HeLa cells by Painter and Schaefer.⁹ The data reported in the present paper seem different from those of Tsukada *et al.*,¹⁸ insofar as we have observed a clearly bimodal distribution of the size of the newly-synthesized DNA, at pulse times longer than one minute. We think, therefore, that the objections raised by Lehmann and Ormerod¹⁹ do not apply to our work,

since random shearing could not produce two distinct size distributions of labeled molecules.

The phenomenon is analogous to the one observed in microorganisms, the size of the pieces being somewhat smaller than that observed in *Escherichia coli* by Okazaki *et al.*¹ The approximate number of pieces present per single chain at the growing point can be estimated to be between 5 and 25, to be compared to an estimate of 3 in the *E. coli* system. Whatever the accuracy and the meaning of this difference, it would seem that the structure of the DNA at the growing point is similar in mammalian cells and in bacteria.

Several points remain to be established before drawing any conclusion as to the detailed structure of DNA at the growing point in human cells; in particular, the possibility of artifacts during the extraction has not yet been completely ruled out in this system. In view of the difficulties encountered in the extraction of the newly polymerized DNA in neutral conditions, we are left with some doubt about the state of the template around the growing point. Considering the extensive degradation of the DNA that was observed when we used mild extracting conditions and the necessity to use more drastic treatment in order to avoid nuclease action, we assume that, in the latter case, denaturation of the newly synthesized DNA occurred. We tentatively conclude then that the most likely explanation for our inability to observe the pulse-labeled DNA attached by hydrogen bonds to the template DNA is the inadequate extraction technique. More work remains necessary to clarify this point.

In our laboratory (unpublished experiments) we have determined the existence of a DNA polymerase and of a polynucleotide ligase activity in extracts of the EUE cells; the description of the properties of these enzymes could give support to the model implied in the study here described. Also, the determination of the polarity of chain growth *in vivo* could help decide on the validity of the observations reported here.

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