Intracellular Location and Kinetics of Complex Formation Between Simian Virus 40 T Antigen and Cellular Protein p53

FLORENCE I. SCHMIEG AND DANIEL T. SIMMONS*

School of Life and Health Sciences, University of Delaware, Newark, Delaware 19716

Received 14 June 1984/Accepted 3 August 1984

The intracellular location and kinetics at which the simian virus 40 T antigen and the cellular protein p53 associate with one another were determined for simian virus 40-transformed mouse (215) and rat (14B) cells. Cells were labeled under pulse-chase conditions and fractionated into nuclear and cytoplasmic components, and the proteins were immunoprecipitated with monoclonal antibodies (pAb 416, 101, and 122). We found that newly made T antigen and p53 migrated to the nucleus of these cells independently; that is, in an uncomplexed form. Newly made p53 was transported to the nucleus more rapidly than T antigen in both cell lines and formed a complex with a mature form of T antigen recognizable by pAb 101. This association was very rapid in both cell lines (t1/2, 5 to 15 min). In contrast, the time course of complex formation between newly made T antigen and p53 in the nucleus varied with the ratio of T antigen to p53 of the cell line studied. In 215 cells, where the ratio was 3.6, the kinetics were quite slow (t1/2, 30 min), whereas in 14B cells, where the ratio was 1.7, they were quite rapid (t1/2, 5 min). We suggest that a competition between newly made and uncomplexed T antigen for the p53 in the nucleus is the major determinant of the rate of complex formation for newly made T antigen.

Our studies indicate that this macromolecular interaction is extremely dynamic.

Mammalian cells infected or transformed by the DNA tumor virus simian virus 40 (SV40) produce a phosphoprotein, p53, that complexes stably to the SV40-encoded tumor (T) antigen (15, 17, 24). This same p53 protein has also been found complexed to the adenovirus early region 1b, 55,000-molecular-weight protein (35) and is found in elevated amounts in cells transformed by retroviruses (8, 34) and Epstein-Barr virus (1). The protein also accumulates in spontaneously transformed and chemically transformed cells (7, 8, 33) and in cells of embryonic origin such as embryos and embryonic carcinoma cells (20, 30).

p53 is found in very low amounts in normal, established cell lines such as 3T3 and BSC-1 (21, 36) and is very unstable in these lines (29, 31), having a half-life of 30 min to 1 h. However, in both transformed cells and cells of embryonic origin, the stability of p53 is greatly increased (5, 31).

The function of p53 is unknown. The protein is species specific and is highly conserved through evolution (25, 36, 37), suggesting that p53 plays an indispensable role in the cell. Milner and Milner (28) have shown that in lymphocytes, the levels of p53 increase after mitogenic stimulation. Antibodies to p53, when microinjected into the nuclei of quiescent 3T3 cells, inhibit serum-induced cell DNA synthesis (26, 27), suggesting a possible role for the protein in the growth regulation of normal cells. The association of p53 with SV40 and adenovirus tumor antigens, which are required for stable transformation (11, 16, 23, 32), suggests that p53 may also participate in one of the steps leading to, or in the maintenance of, transformation of normal cells.

Therefore, it is important to understand the molecular events involved in complex formation between these tumor antigens and the cellular protein p53. Other investigators have determined that the mature SV40-specific complex sediments between 24S and 28S in sucrose gradients (12, 24), and we have shown that this complex is composed of four molecules of T antigen and four to five molecules of p53 (10). The complex first forms as a 16S intermediate (12). However, little else is known about this process. In this study, we have examined the kinetics of formation of this complex in two lines of SV40-transformed cells. We have identified the nucleus as the intracellular site where complexing occurs, and we have determined the approximate times at which T antigen and p53 are transported from the cytoplasm to the nucleus.

MATERIALS AND METHODS

Cell lines. SV40-transformed mouse 215 cells were obtained from an early-passage cloned normal AL/N mouse embryo line transformed by SV40 and isolated by cloning single cells (39).

SV40-transformed rat cell line 14B was isolated by W. Topp and described in Botchan et al. (2).

Cell fractionation. In situ fractionation was performed as follows. Labeled cell cultures were washed twice with cold phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH2PO4, 140 mM NaCl, 13 mM Na2HPO4 [pH 7.4]) and once with freshly made buffer containing 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 6.6), 10% glycerol, 5 mM MgCl2, 3 mM CaCl2, 1 mM dithiothreitol, and 250 μg of phenylmethylsulfonyl fluoride per ml. Cells were lysed on ice in the same buffer containing 1% Nonidet P-40 (cell lysis buffer) without scraping. Under these conditions, the nuclei remained attached to the flask. After 15 min, the solubilized cytoplasmic material was removed and the nuclei were washed once with the same buffer and lysed in situ in buffer containing 20 mM Tris-hydrochloride (pH 9.0), 150 mM NaCl, 1% Nonidet P-40, and 20 mM EDTA (nuclei lysis buffer). The nuclei lysis buffer was removed, the flask was washed once with the same buffer, and the wash was added to the nuclear fraction. The cytoplasmic and nuclear fractions were clarified by centrifugation at 2,800 × g for 10 min and dialyzed at 4°C for 1.5 h and 4 h, respectively, against buffer containing 20 mM Tris-hydrochloride (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40 (6). The samples were stored at -80°C.

A second fractionation scheme was used in some experiments to generate purified nuclei. The cytoplasmic fraction was prepared as described above; nuclei were then scraped.

* Corresponding author.
into cell lysis buffer and homogenized extensively until judged clean by phase-contrast microscopy (ca. 1,500 strokes of a type B Dounce homogenizer). The solution was centrifuged at 2,800 \( \times g \) for 10 min, and the supernatant was removed and dialyzed for 1.5 h as described above. Nuclei were suspended in nuclei lysis buffer, vortexed extensively, and incubated on ice for 15 min. The sample was then subjected to ultrasound for 60 s at 0°C with a Branson 12 Ultrasound Cleaner (117 V) to completely disrupt the nuclei.

The cytoplasmic and nuclear fractions were clarified and dialyzed as before.

Cell fractionation and immunoprecipitation. Cultured cells were labeled for either 2 h or 5 min with L-\([^{35}S]\)methionine (40 to 200 \( \mu \)Ci/ml) in Eagle medium lacking unlabeled methionine and containing 2% dialyzed fetal bovine serum. When the label was to be "chased," cultures were washed two times with Eagle medium containing methionine and 2% fetal bovine serum and incubated at 37°C in the same medium for the time indicated. Cells were either fractionated as described above or lysed in buffer containing 20 mM Tris hydrochloride (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40 (6) for preparation of total cell lysates.

Immunoprecipitation reactions were performed as previously described (19).

Acrylamide gel electrophoresis and densitometry. Acrylamide slab gel electrophoresis of sodium dodecyl sulfate-denatured proteins was performed as previously described (37). For detection of labeled proteins, gels were soaked in 10 volumes of 1 M sodium salicylate for 45 min (4), dried, and exposed to Kodak XAR-5 film for 3 to 42 days at \(-80^\circ\)C.

Films were scanned with a Densicord electrophoresis densitometer (no. 552, PhotoVolt Corp., New York).

RESULTS

Fractionation of nuclear and cytoplasmic proteins. For studying the fates of newly made T antigen and p53, and in particular for following their transport into the nucleus, it was important to use a cell line in which, at steady state, these two proteins were known to be located predominantly in the nucleus. We chose a line of SV40-transformed mouse cells isolated by Winterbourne and Mora (39) that synthesize substantial amounts of T antigen and p53 (215 cells), and in which very little of either protein is found in the cytoplasm under steady-state conditions (22). To correctly identify the intracellular location of labeled T antigen and p53 at any one time, the cells had to be fractionated under conditions that prevented the loss of proteins from the nucleus. For this reason, we developed an in situ fractionation procedure similar to that described by Staufenbiel and Deppert (38), but with a cell lysis buffer containing Mg\(^{2+}\) and Ca\(^{2+}\) ions (see above). An in situ approach was preferred to other protocols that required scraping and homogenizing of cells (9, 22) because it afforded greater ease in the handling of large numbers of flasks and allowed more efficient recovery of nuclear and cytoplasmic material. In this procedure, all of the cells were disrupted after a few minutes in the lysis buffer (as determined by phase microscopy), but the nuclei and some cytoskeleton remained attached to the flask. The attached nuclei were then lysed in buffer containing 20 mM EDTA, which was necessary for complete lysis and for the solubilization of nuclear proteins.

To test this fractionation scheme, SV40-transformed mouse 215 cells were labeled for 2 h with L-\([^{35}S]\)methionine and fractionated as described above, and the nuclear and cytoplasmic fractions were incubated with pAb 416, a monoclonal antibody that recognizes all forms of T antigen (14); with pAb 101, a monoclonal antibody that preferentially recognizes oligomerized forms of T antigen (3, 13); or with pAb 122, and antibody directed against mouse p53 (13). In this and subsequent experiments, immunoprecipitations were performed in large antibody excesses to ensure that all antigens had reacted (10). The amount of antibody that was required was determined, in each case, by an antibody saturation experiment. Labeled, immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography, as described above. T antigen and p53 were found almost exclusively in the nuclear fraction (Fig. 1, lanes 2 to 4), indicating that leaching of nuclear proteins into the cytoplasmic fraction was minimal. In all likelihood, most of the soluble cytoplasmic proteins were removed in the initial cell lysis buffer, because ca. 70% of the incorporated methionine label in the cell was associated with this fraction (see also Fig. 4).

For the experiments described in this report, it was necessary to perform pulse-chase experiments with a very short pulse time (5 min) (see below). Since the fractionation scheme does not produce purified nuclei, we first needed to know whether T antigen and p53 labeled in a pulse and found in the nuclear fraction were indeed truly nuclear, and not associated with cytoskeletal components attached to the nuclei. For an answer to this question, 215 cells were pulse-labeled for 5 min with L-\([^{35}S]\)methionine and lysed as described above. After the removal of the cytoplasmic fraction, the nuclei were scraped into the same buffer and homogenized extensively until judged clean by phase microscopy. The nuclei were pelleted, the homogenate was removed, and the nuclei were then incubated in lysis buffer containing EDTA. All three fractions were subjected to immunoprecipitation with pAb 416 and pAb 122 as described above. Virtually no T antigen or p53 was associated with the

FIG. 1. Association of T antigen and p53 with the nucleus of in situ-fractionated SV40-transformed mouse 215 cells. The 215 cells were labeled for 2 h with L-\([^{35}S]\)methionine at a concentration of 50 \( \mu \)Ci/ml. Cells were subjected to in situ fractionation as described in the text, and fractions were incubated with nonimmune medium, pAb 416, pAb 101, or pAb 122, and then with Staphylococcus aureus. Precipitated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 13% gels, and the labeled bands were detected by fluorography. Lanes 1 to 4 represent nuclear material, and lanes 5 to 8 represent cytoplasmic material. Lanes 1 and 3: nonimmune medium. Lanes 2 and 6: pAb 416. Lanes 3 and 7: pAb 101. Lanes 4 and 8: pAb 122.
homogenized fraction (Fig. 2, lanes 5 and 6). Nuclei treated in this way were for some reason very difficult to lyse, and consequently the amounts of labeled T antigen and p53 recovered from the nuclear fraction were quite low (Fig. 2, lanes 2 to 4). Nevertheless, the absence of any detectable T antigen or p53 in the cytoskeletal fraction indicated that the proteins found in the nuclear fraction after in situ fractionation were truly nuclear.

**Location and kinetics of complex formation.** For calculation of the kinetics of complex formation, it was important to label cells very briefly such that most of the labeled T antigen and p53 were not complexed. Preliminary experiments showed that 5 min of labeling was adequate for this purpose but that 10 min was too long. We therefore pulse-labeled cells for 5 min with L-[35S]methionine and estimated the proportions of labeled T antigen and p53 that were complexed. To assess this accurately without possible artifact of the fractionation protocol, 215 cells were lysed in the absence of Ca^{2+} and Mg^{2+} to solubilize both nuclear and cytoplasmic proteins. The extract was incubated with pAb 416, pAb 101, and pAb 122 in separate reactions. After 5 min of labeling, the majority of newly made p53 and T antigen molecules were not complexed (Fig. 3). (Compare the relative amounts of p53 in lanes 4 and 2 and of T antigen in lanes 2 and 4.) From densitometric tracings of the X-ray film, we determined that less than 1% of pulse-labeled T antigen and ca. 13% of pulse-labeled p53 were complexed after 5 min. This same proportion of the labeled p53 in the extract (13%) was precipitated with pAb 101 (Fig. 3, lane 3). Since pAb 101 does not react with newly synthesized T antigen (3), pulse-labeled p53 associated with previously synthesized T antigen that had matured to a form recognizable by pAb 101. This is consistent with previous observations of Carroll and Gurney (3).

Pulse-chase experiments were performed for a determination of the intracellular location and kinetics of complex formation of T antigen and p53. Five subconfluent flasks of 215 cells were pulsed for 5 min with L-[35S]methionine and chased for 0, 5, 15, 30, or 120 min. After the chase, the cells were fractionated in situ as described above. Fractions from each time point were reacted with pAb 416, pAb 101, and pAb 122, and the precipitated labeled proteins examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. A complex between T antigen and p53 was not detectable in the cytoplasmic fraction at any time point, although much longer exposures did show a minor amount of complexed labeled p53 in the cytoplasm (Fig. 4). However, the vast majority of complexed, labeled T antigen and p53 was found in the nucleus.

The amount of radioactivity in each T antigen and p53 band in Fig. 4 was estimated by densitometry. Transport of p53 into the nucleus was very rapid (Table 1). Virtually 100% of labeled p53 was present there after a 5-min pulse. Therefore, p53 has an extremely high affinity for the cell nucleus. T antigen was transported to the nucleus more slowly (Table 1). Approximately 33% of newly made T antigen was present in the nucleus after 5 min, and 80 to 85% was present after a subsequent 5-min chase. This amount increased to 99% by 120 min.

The kinetics of complex formation were also different for the two proteins (Table 2). Newly synthesized p53 complexed very rapidly to the unlabeled T antigen already present in the nucleus, whereas newly synthesized T antigen became complexed to p53 much more slowly. By comparing the amounts of p53 precipitated with pAb 416 (Fig. 4, lanes 2) and with pAb 122 (Fig. 4, lanes 4), we determined that after a 5-min chase the majority (77%) of the labeled p53 was already complexed to T antigen (Table 2). In this experi-

![FIG. 2. Absence of T antigen and p53 from the cytoskeletal fraction. Cells were labeled for 5 min with L-[35S]methionine at a concentration of 250 μCi/ml and fractionated as described in the text. Fractions were incubated with nonimmune medium, pAb 416, or pAb 122, and the precipitates were subjected to electrophoresis (see the legend to Fig. 1). Lanes 1 to 3 represent nuclear material. Lanes 4 to 6 represent cytoskeletal material homogenized free from the nuclei; lanes 7 to 9 represent cytoplasmic material. Lanes 1, 4, and 7; nonimmune medium. Lanes 2, 5, and 8: pAb 416. Lanes 3, 6, and 9: pAb 122.](http://jvi.asm.org/)
ment, ca. 14% of newly made p53 was in complex form after a 5-min pulse (Table 2). This agrees well with the data obtained from unfractionated 215 cells (Fig. 3, lanes 2 and 4). A drop in the amount of complexed labeled p53 was seen after a 30-min chase (Table 2). We have observed a similar drop in other experiments (data not shown). Displacement of the labeled complexed p53 with unlabeled p53 or T antigen between 15 and 30 min in the chase may explain this. The fraction of complexed p53 present after a 120-min chase was ca. 61% (Table 2). This agrees well with the results obtained after a continuous 2-h label (Fig. 1, lanes 2 and 4).

It proved difficult to obtain clean immunoprecipitates of nuclear preparations labeled during a 5-min pulse. The generally high background of labeled proteins (Fig. 4, 0 min, lanes 2 to 4) disappeared for the most part after a 5-min chase and therefore was not due to the extraction and immunoprecipitation conditions that were used, but probably reflects the fact that nascent, unfinished polypeptides were labeled. The cytoskeleton that was still attached to the nuclei could possibly have contained polysomes that would contribute to the increase in background. Due to this increased background, however, quantitation of the labeled bands in the 5-min pulse sample by densitometry was not very accurate, and variable results were obtained in repeated experiments. However, all of these experiments showed quite conclusively that most of the newly made p53 complexed to T antigen in the nucleus by 5 to 10 min.

In the 215 cells, newly made T antigen complexed to p53 much more slowly, taking 2 h to reach the maximum level of complexing observed in these experiments (ca. 32%) (Table 2 and Fig. 4). This slow rate may reflect the need for T antigen to undergo posttranslational changes before becoming complexed to p53 (12), or it may be due to the large ratio of T antigen to p53 in the nucleus of these cells. To test this latter point, we repeated the pulse-chase experiment with another SV40-transformed cell line (rat 14B) containing a much smaller ratio of T antigen to p53 than that in the 215 cell line (10). After a long label, the T antigen-to-p53 ratio in 14B cells was ca. 1.7, compared with 3.6 in 215 cells. Therefore, 14B cells contain a much smaller pool of preexisting uncomplexed T antigen in the nucleus to compete with newly made T antigen for binding to p53. Nascent T antigen in 14B cells complexed far more rapidly than in 215 cells, taking only 5 min for 46% to complex to p53 (Table 3). Also, a greater proportion of the T antigen was in complex form in these cells, with 76% complexed after 2 h (Table 3). In 14B cells, more than half of the T antigen that will become complexed in the cell does so within 5 min. This contrasts with the behavior of T antigen in 215 cells, in which 30 min was required to reach this same relative amount of complexing (Table 2). Therefore, it appears that the rate of complex formation between newly made T antigen and p53 is influenced more by the ratio of T antigen to p53 in the nucleus than by a requirement that T antigen first undergo posttranslational modifications.

Newly made p53 complexed to T antigen slightly more slowly in 14B cells than in 215 cells, taking ca. 15 min to reach 48% complexing. This was nearly the maximum level (55%) observed in these cells (Table 3). The difference in the kinetics at which p53 complexes to T antigen in these two cell lines appears to be small.

p53 entered the nucleus more slowly in 14B cells than in 215 cells (100% in 20 and 5 min, total time, respectively) (Tables 1 and 4). T antigen, however, entered the nucleus with similar kinetics in both cell lines (Tables 1 and 4).

New protein bands. Several previously uncharacterized proteins were detected in immunoprecipitates of pulse-labeled samples (Fig. 3 and 4). Two proteins with apparent

| TABLE 2. Percentage of newly made T antigen and p53 in complex versus length of chase in 215 cells |
| --- | --- | --- |
| Time (min) | T % in complex | p53 % in complex |
| 0 | 0.0 | 14 |
| 5 | 2.3 | 77 |
| 15 | 3.2 | 73 |
| 30 | 19.2 | 33 |
| 120 | 32.0 | 61 |

| TABLE 3. Percentage of newly made T antigen and p53 in complex versus length of chase in 14B cells |
| --- | --- | --- |
| Time (min) | T % in complex | p53 % in complex |
| 0 | 3 | 6.5 |
| 5 | 46 | 28.0 |
| 15 | 42 | 48.0 |
| 30 | 60 | 53.0 |
| 120 | 76 | 55.0 |
molecular weights of 71,000 and 64,000 were precipitated with pAb 416 (Fig. 3 and 4, bands A and B) from the cytoplasmic fraction of pulse-labeled cells. These bands declined in intensity with increasing chase times and were totally absent by 2 h (Fig. 4; other data not shown). Peptide maps of these two proteins indicated that they were truncated forms of T antigen (data not shown).

A third protein with an apparent molecular weight of 88,000 (Fig. 3, band Z) was precipitated with pAb 122. However, it was not precipitated with another monoclonal antibody to p53, pAb 421 (data not shown). This suggests that it is a cellular protein unrelated to p53.

**DISCUSSION**

**Nuclear location of complex formation.** In this series of experiments, we determined that the nucleus is the site at which T antigen complexes to p53 in both 215 and 14B cells. Since T antigen is a nuclear protein that plays a necessary role in the initiation and maintenance of transformation in SV40-transformed cells (23, 32), it is reasonable to consider the nucleus as an important site of T antigen function during malignant transformation. Similarly, the affinity of p53 for the nucleus and the implication of a role for p53 in growth regulation of normal cells (26, 27) suggests that the nucleus is an important target area for p53 regulatory effects. Therefore, localization of the nucleus as the major site of complexing between these two important proteins lends support to the idea that p53 is deregulated in a manner when SV40 transforms the cell, and that this deregulation is important for either the initiation or the maintenance of the transformed phenotype in these cells.

The entry of p53 and T antigen into the nucleus is very rapid. By 5 min, substantial amounts of both proteins are found there in both 215 and 14B cells. The arrival of p53 into the nucleus of 215 cells is most impressive. Virtually 100% is present after a 5-min pulse. This suggests that p53 may have an important role to play in the nucleus of these cells, or that these cells possess an extremely efficient transport system, or both. p53 entry into 14B cell nuclei is also rapid, but slower than in 215 cells.

The different rates of nuclear transport for T antigen and p53 may reflect a difference in the amino acid sequence in the two proteins that signals for nuclear transport. This signal has been mapped for SV40 T antigen to the NH2-terminal end of the protein (18). The transport signal for p53 is not known, but it must be extremely strong, as evidenced by the rapid kinetics by which p53 enters the nucleus.

**Kinetics of complex formation.** We also determined that newly made p53 associates with T antigen very rapidly (5 to 15 min) in both cell lines studied. This is consistent with the results obtained by Greenspan and Carroll (12). The kinetics of complex formation of newly made T antigen, however, appeared to be dependent upon the relative ratio of T antigen to p53 in the cell, suggesting that newly transported nuclear T antigen competes with preexisting T antigen for p53. Other investigators have suggested that T antigen must undergo a posttranslational modification as a prerequisite to complex formation (3, 12). Our finding that, in 14B cells, T antigen is substantially complexed (46%) in 5 min argues against this requirement. It is our observation that T antigen does mature to a form recognizable to pAb 101, as has been previously reported (3, 12). However, this maturation occurs quickly (1/2, 15 min) as evidenced by the appearance of labeled T antigen bands in the pAb 101 lanes of Fig. 4, and does not correlate with the slow kinetics at which newly made T antigen complexes to p53 in 215 cells (Table 2).

Previous work has suggested that the majority of the p53 in 215 cells is in complex form (5). This is consistent with our observation that 77% of newly made p53 is in complex form after a 5-min chase in 215 cells. It is interesting that this number drops in the longer chases, stabilizing at ca. 55 to 60% after 2 h (Table 2). We feel that these values are accurate because all antibodies used in the immunoprecipitation reactions were in large excess, as determined by antibody titration experiments. We propose that the complex between p53 and T antigen is a dynamic association, capable of exchanging monomeric (or perhaps tetrameric) units with other complexes or with uncomplexed molecules present in the nucleus. In 215 cells, the initial complexing of newly made p53 is very rapid (5 min) and reaches a high level (77%) due to the presence of substantial amounts of uncomplexed T antigen available for complexing (70%). During the longer chase periods, however, new, unlabeled p53 and T antigen molecules continue to be made and enter the nucleus, perhaps displacing some of the labeled complexed p53 molecules.

In 14B cells, whose T antigen/p53 ratio is lower, complexing of newly made p53 molecules is a bit slower (15 min) and reaches a maximum level of only 55%. This could reflect the smaller pool of uncomplexed T antigen (30%) or the slower transport of newly made p53 or both in these cells. The lower amounts of uncomplexed T antigen in these cells could also explain why the level of complexed p53 does not drop over the chase period, as was seen in 215 cells. If T antigen can displace the p53 in the complex, this effect would be less of a factor in cell lines with a smaller uncomplexed T antigen pool.

It is also possible that the different results obtained with mouse 215 cells and rat 14B cells may simply reflect a species-dependent difference in T antigen-p53 complexing. However, we feel this is unlikely because the p53 molecules found in mouse and rat are structurally similar and these proteins are highly conserved (36, 37).

Taken together, these studies indicate that the association between T antigen and p53 is an extremely rapid and dynamic process. The role of tumor antigen-p53 complexes in SV40- and adenovirus-transformed cells and of p53 in normal and in non-virus-transformed cells needs further investigation.

**ACKNOWLEDGMENTS**

This work was supported by a University of Delaware Research Foundation grant and by a Public Health Service biomedical research grant.

**LITERATURE CITED**


2. Botchan, M., W. Topp, and J. Sambrook. 1976. The arrange-