Cell Cycle Dependent Transcription of SV40 DNA in SV40-Transformed Cells†

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ABSTRACT: A high degree of synchrony of growth was achieved with Simian virus 40 (SV40) transformed epitheloid rat liver cells (WIRL-3C SV) using a thymidine block followed by colcemid arrest. The synchronously growing cells were pulse labeled with [^{3}H]uridine at different stages of the cell cycle and RNA was isolated and hybridized to SV40 DNA I. Only RNA synthesized during the S-phase of the cell cycle showed significant complementarity to the viral DNA. Chromatin was isolated at different stages from these synchronously growing cells and used as template for synthesis *in vitro* of RNA by *Escherichia coli* RNA-polymerase. The purified RNA samples were annealed to SV40 DNA. The transcription product from chromatin of cells harvested during the S-phase hybridized to SV40 DNA. Thus, *in vitro* transcription from chromatin reflects the pattern of SV40 specific RNA synthesis in intact cells.

Direct evidence that SV40[1] DNA associates with the DNA of the host genome during infection of nonpermissive cells has been obtained (Hirai *et al.*, 1971). In SV40-transformed cells, the viral DNA cosediments with high molecular weight cellular DNA in an alkaline stable form (Sambrook *et al.*, 1968). Several copies of SV40 DNA may be associated with one transformed cell, although the exact number of gene equivalents per cell is still a matter of dispute. There is, however, no correlation between the extent of virus specific transcription and the number of virus genomes per cell. The expression of the transformed phenotype is linked to the presence of RNA coded by that strand of SV40 DNA which is responsible for the early viral functions.

These results prompted us to look to the question of whether or not the host cell exercises a control on the transcription of the integrated viral genome which may be expressed as a synchrony of viral RNA synthesis during the cell cycle. A transformed cell line was selected for study which could be synchronized to a high degree. The RNA synthesized at the different stages of the cell cycle was labeled by [^{3}H]uridine and subsequently hybridized to SV40 DNA. Transcription of the SV40 DNA occurs at characteristic times in the cell cycle.

Isolated chromatin is endowed to a certain extent with regulatory properties similar to those of intact cells. It has been demonstrated that chromosomal proteins change their pattern of phosphorylation and thiol content throughout the cell cycle (Ord and Stocken, 1968; Shepherd *et al.*, 1971). In SV40 transformed mouse cells asymmetric transcription of SV40 DNA occurs from chromatin with *Escherichia coli* RNA polymerase similar to the transcription in intact cells (Astrin, 1973; Shih *et al.*, 1973). An attempt to elucidate the control mechanisms of the SV40 specific transcription was undertaken by using isolated chromatin of various proliferative stages of synchronized WIRL-3C-SV cells as template for RNA synthesis by *E. coli* DNA-dependent RNA polymerase. The fraction of SV40 specific sequences in the various samples of this *in vitro* synthesized and labeled RNA was determined by hybridization to SV40 DNA. The results obtained agreed essentially with those obtained in intact cells.

Materials and Methods

Cells. WIRL-3C-SV cells originate from epitheloid rat liver cells by transformation with SV40 (strain Rh 911) and were kindly supplied by Dr. L. Diamond. Cells were cultured in Basal Medium Eagle (BME) supplemented with 10% fetal bovine serum. For synchronization experiments the serum was dialyzed against BME prior to use.

Virus. SV40 (strain Rh 911) was grown in roller cultures of monkey kidney (CV-1) cells, purified (Swetly *et al.*, 1969), and banded twice in CsCl density gradients, and type I viral DNA was isolated free of cellular DNA contamination by zonal centrifugation in alkaline sucrose gradients (Barbanti-Brodano *et al.*, 1970). The closed circular form I was nicked with DNase (Vinograd *et al.*, 1965) and adsorbed to membrane filters (Gillespie and Spiegelman, 1965) to give 2 μg of DNA immobilized per filter.

Cell Fractionation. Cells were lysed in the presence of NP-40 and nuclei isolated as previously reported (Barbanti-Brodano *et al.*, 1970). DNA was prepared from washed WIRL-3C-SV nuclei. Chromatin was isolated in the absence of EDTA from washed nuclei (Dahmus and McConnell, 1969) by homogenizing the nuclear pellet in 20 volumes of cold 0.01 M Tris buffer (pH 8.0) in a Teflon homogenizer and further stirring for 30 min on a magnetic stirrer. Chromatin was then precipitated by centrifugation at 12,000*g* for 15 min, washing three times by suspension in cold 0.01 M Tris buffer (pH 8.0), and sedimentation at 12,000*g*. DNA equivalents in chromatin were determined by the diphenylamine reaction as described by Burton (1956) using as a standard rat liver DNA. DNA-dependent RNA polymerase was prepared from *E. coli* according to the method of Chamberlin and Berg (1962) and purified to step 4.

Cell Cycle Analysis. WIRL-3C-SV cells were grown on coverslips in petri dishes and exposed to tritiated thymidine (2 μCi/ml and 18 Ci/mmol) for 15 min at different times, and

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[1] Abbreviation used is: SV40, Simian virus 40.

then changed to fresh medium containing 0.02 mM unlabeled thymidine. At hourly intervals one coverslip was removed and prepared for autoradiographic analysis (Schmid and Carnes, 1965). The fraction of labeled mitoses was determined (Quastler and Sherman, 1959; Mendelsohn and Takahashi, 1971).

Cell Synchronization. Synchronization of cells cultured in roller flasks (670 cm^2 growth area) was initiated when cell growth was in its exponential phase. All procedures were carried out strictly at 36°. Two methods were used: *double thymidine block* and thymidine block followed by colcemid arrest. The method of *double thymidine block* involved exposure of cells to 3×10^{-3} M thymidine in BME supplemented with 10% dialyzed fetal bovine serum for 9 hr, washing of the cell monolayers with BME, and release from the block for 8 hr in the presence of 10^{-5} M *2'-deoxycytidine.* A second cycle of thymidine block was carried out under identical conditions as the first block and was succeeded by a release in the presence of 10^{-5} M 2'-deoxycytidine.

For the combination of *thymidine block* and *colcemid arrest* the first cycle of thymidine block was carried out as described above. After release for 4 hr in the presence of 10^{-5} M 2'-deoxycytidine the cells were arrested for 4 hr with 0.15 μg/ml of colcemid. The colcemid was removed, the cells were carefully washed in order not to detach them from the glass, and fresh BME with 10% fetal calf serum was added after the release from the colcemid arrest. Synchrony of DNA synthesis in cells treated with thymidine–colcemid was tested by pulse labeling with 0.1 μCi/ml of [^{3}H]thymidine for 30 min at various intervals after release from the colcemid arrest.

Radioactive measurements were carried out by liquid scintillation counting in a toluene–Liquifluor system. Determination of RNA and DNA concentrations was carried out by colorimetric methods (Shatkin, 1969). [6-^{3}H]Thymidine, [5,6-^{3}H]uridine, and uridine 5'-[5-^{3}H]triphosphate were obtained from New England Nuclear and had specific activities of 48, 35.8, and 26 Ci/mmol, respectively. Buffers used were: NT buffer, 0.14 M NaCl–0.01 M Tris-Cl (pH 7.4); 1 × SSC, 0.15 M NaCl–0.015 M sodium citrate.

Results

Characterization of Cell Line WIRL-SV-3C. In order to determine if the transcription of integrated SV40 DNA in transformed cells is dependent on the stage of the cell cycle, several criteria were applied to the choice of the cell line. No infectious SV40 could be detected in concentrated supernatants of WIRL-3C-SV cells by plaque titration (Swetly *et al.,* 1969). The presence of the intact SV40 genome in the cells could be demonstrated by rescue of infectious virus after Sendai virus-mediated fusion (Koprowski *et al.,* 1967) between WIRL-3C-SV cells and monkey kidney (CV-1) cells. No direct evidence is available for covalent integration of SV40 DNA into the genome of WIRL-3C-SV cells. However, no infectious SV40 DNA was detected in the low molecular weight DNA of the transformed cells after selective extraction (Hirt, 1967) when tested on susceptible CV-1 cells (Swetly *et al.,* 1969). The synthesis of SV40-specific RNA was proven by hybridization to SV40 DNA of RNA from randomly growing WIRL-3C SV cells. Radioactive labeling, extraction of RNA, and hybridization were carried out as described for synchronized cells in Figure 2. An aliquot of 0.013% from the radioactivity incorporated into RNA was complementary to SV40 DNA. All WIRL-3C-SV cells displayed immunofluorescence specific for SV40 induced T-antigen.

To determine optimum conditions for cell synchronization, an autoradiographic cell cycle analysis was carried out. The results show that the transit time through the S-phase (7–8 hr) is shorter than the sum of transit times through the other compartments of the cell cycle: G2 + G1 + M (G1 = 4–5, G2 = 3, M = 0.7 hr, respectively) indicating that the thymidine block is a successful method of cell synchronization.

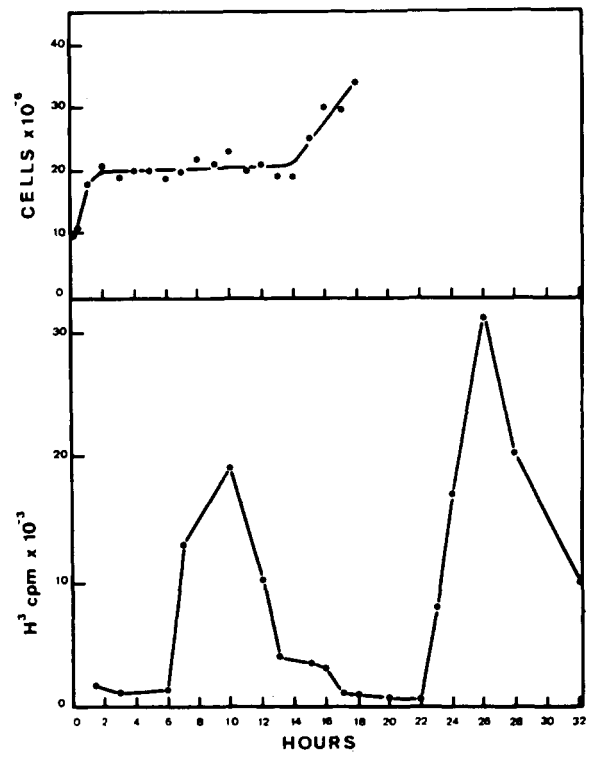

FIGURE 1: Synchronization of WIRL-3C-SV cells by thymidine–colcemid treatment; determination of cell number and DNA synthesis after release from the block. (a) In the upper panel the number of cells per culture at various intervals after release from the colcemid arrest is shown. (b) The lower panel illustrates the amount of [^{3}H]thymidine incorporated into acid-precipitable material during 30-min pulses at different time intervals after release from the colcemid block.

Synchronized WIRL-3C SV Cells Synthesized SV40-Specific RNA Mainly during the S-Phase of the Cell Cycle. Two methods for synchronization were used in these experiments: the double thymidine block (Puck, 1964) and a single thymidine block followed by a colcemid arrest. Both methods gave a synchrony of cell growth in the initial steps of between 80 and 90% with respect to the initiation of DNA synthesis. Although both methods gave similar results in the hybridization experiment, we preferred the thymidine–colcemid block because the length of the G1 phase after the double thymidine block was shorter than that determined by cell cycle analysis of randomly growing cells. The colcemid block did not interfere with the viability of the arrested cells and within 1 hr after release the cell number had doubled without occurrence of dead cells (Figure 1). The concentration of colcemid sufficient for metaphase arrest of WIRL-3C-SV cells in stationary cultures is significantly lower (0.025 μg/ml) than that required in roller cultures (0.15 μg/ml). This may be explained by the lower medium/cell ratio in the roller cultures.

Figure 2 shows the results of a hybridization experiment using 7.5×10^6 cpm of RNA from different stages of synchronous WIRL-3C-SV cells and an excess of immobilized SV40 DNA. This method does not determine the absolute amount of SV40-specific RNA sequences synthesized at the indicated interval but rather measures virus-specific RNA synthesis relative to total cellular RNA synthesis during the labeling period. This relative determination of SV40-specific sequences offered the advantage of eliminating errors which result from varia-

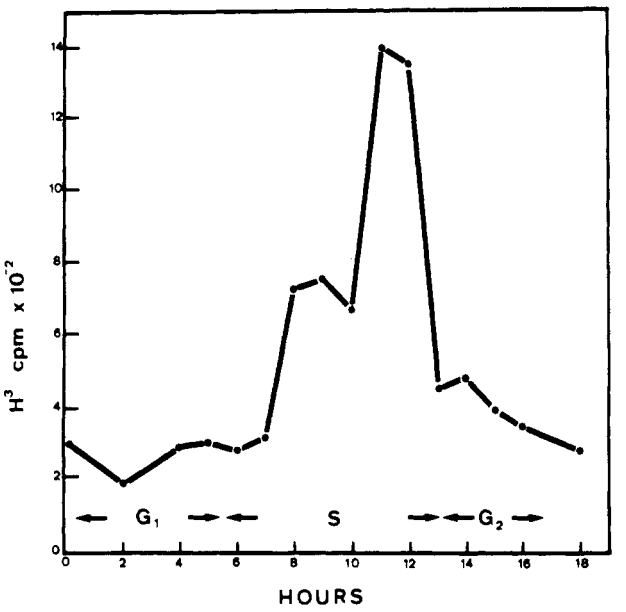

FIGURE 2: Hybridization to SV40 DNA of pulse labeled RNA isolated from synchronized WIRL-3C-SV cells at different stages of the cell cycle. Cells cultured in roller bottles were synchronized by the thymidine-colcemid method. At each 2-hr interval after release from the colcemid arrest the medium was removed from two roller bottles and the cells were exposed for 2 hr to 0.8 mCi of [5,6-^{3}H]uridine in 10 ml of fresh BME containing 10% fetal calf serum. After that period the cells were twice washed with cold sodium phosphate buffered saline and harvested by trypsinization; the total cell RNA was extracted with hot phenol–sodium dodecyl sulfate (Scherrer, 1969) and precipitated with 2 vol of ethanol. The precipitates were dissolved in 2 ml of NT buffer, treated with 10 μg/ml of DNase I (RNase free) and 3×10^{-4} M $MgCl_2$ for 30 min at 30°, again extracted with phenol, filtered through a Sephadex G-75 column, and precipitated with ethanol, and the precipitate was dissolved in 0.1 × SSC. A constant amount of acid precipitable radioactivity (7.5×10^6 cpm) was used for each hybridization set. Prior to hybridization the RNA samples were heated to 100° for 5 min and adjusted to 2 × SSC. Hybridization was carried out with SV40 DNA immobilized on Millipore filters and allowed to proceed at 65° for 18 hr. One vial contained in a total volume of 1 ml: two filters with 2 μg of nicked form I SV40 DNA, two blank filters, 2 × SSC, 0.1% sodium dodecyl sulfate, 7.5×10^6 cpm of WIRL-3C-SV RNA. After the incubation the filters were washed extensively at 65° and treated with 10 μg/ml of pancreatic RNase A and 10 units/ml of T-1 RNase for 1 hr at 36°. The RNase resistant radioactivity bound to the filters was determined. Radioactivity bound to blank filters did not exceed 90 cpm and was subtracted for each determination.

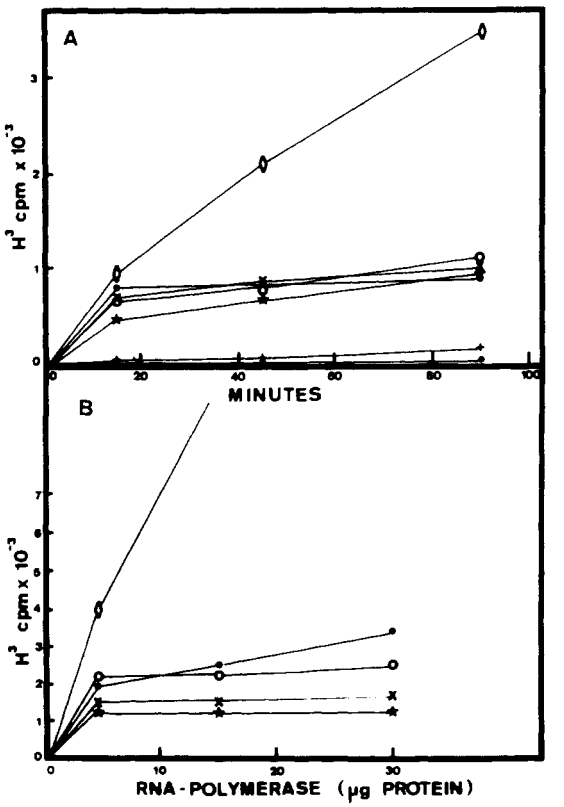

FIGURE 3: (A) Transcription of chromatin from synchronized WIRL-3C-SV cells isolated at different stages of the cell cycle and from WIRL-3C-SV DNA. WIRL-3C-SV cells were cultured and synchronized as described in the legend to Figure 1. At 3-hr intervals following the release from the colcemid block one roller bottle was harvested and the chromatin was isolated from the synchronized cells as described under Materials and Methods. Thus, chromatin was obtained from cells in the phases G1, early S, late S, and G2 of the cell cycle. DNA was isolated from unsynchronized WIRL-3C-SV cells. The transcription *in vitro* of chromatin or DNA was carried out at 36° in a total volume of 1 ml containing 50 μmol of Tris-Cl (pH 8.0), 6 μmol of $MgCl_2$, 100 μmol of KCl, 1 μmol of $MnCl_2$, 0.8 μmol each of guanosine triphosphate, cytidine triphosphate, and adenosine triphosphate, 0.2 μmol of [5-^{3}H]uridine triphosphate (sp act., 25 μCi/μmol), 25 μg of *E. coli* RNA polymerase, 50 μg of WIRL-3C-SV DNA, or 50 μg of DNA equivalents in chromatin. Aliquots of 100 μl were removed from the incubation mixture at intervals shown and the acid precipitable radioactivity was determined as described in the legend to Figure 1. Chromatin isolated during: (★) G1-phase; (*) early S-phase; (×) late S-phase; (○) G2-phase; (◇) DNA; (●) no template; (+) random chromatin without exogenous polymerase. (B) Saturation of rate-limiting concentrations of chromatin from WIRL-3C-SV cells isolated at different stages of the cell cycle with increasing concentrations of RNA polymerase. Chromatin and DNA were prepared as described in A. The transcription *in vitro* was carried out in a total volume of 0.2 ml for 10 min at 36°. The mixture contained 10 μmol of Tris-Cl (pH 8.0), 1.2 μmol of $MgCl_2$, 20 μmol of KCl, 0.2 μmol of $MnCl_2$, 0.16 μmol each of guanosine triphosphate, cytidine triphosphate, and adenosine triphosphate, 40 nmol of [5-^{3}H]uridine triphosphate (sp act., 25 μCi/μmol), 1.4 μg of WIRL-3C-SV DNA, or 1.4 μg of DNA equivalents of WIRL-3C-SV chromatin. *E. coli* RNA polymerase was added at concentrations of 5, 15, and 30 μg of protein, respectively. The cold acid precipitable radioactivity was determined. Chromatin isolated during: (★) G1-phase; (*) early S-phase; (×) late S-phase; (○) G2-phase; (◇) DNA.

tions in cell number between the different samples, in quantitative yield in the RNA extraction procedure, and possible fluctuations of the intracellular UTP pool throughout the cell cycle. The results show that during late S-phase 0.017% of the total RNA is complementary to SV40 DNA, whereas during G1 and G2 only 0.005% is SV40 specific, indicating a more than threefold increase during the S-phase. SV40 RNA synthesis in these transformed cells thus seems to be dependent on the state of the cell cycle, proceeding preferentially during the late S-phase.

Chromatin Isolated during Different Stages of the Cell Cycle Does Not Vary in Its Template Activity. We have attempted to investigate whether the chromatin structure varies throughout the cell cycle in respect to availability of DNA as a template for transcription *in vitro* by *E. coli* RNA polymerase. The results are summarized in Figure 3 and show the synthesis *in vitro* of RNA using chromatin isolated from synchronized WIRL-3C-SV cells at different stages of the cell cycle and rate limiting concentrations or saturating amounts (Figure 3A) of *E. coli* RNA polymerase. Judging from total RNA synthesis no major differences were observed in the kinetics of transcription of the different chromatin samples. Thus, no significant fluctuations in the gross accessibility of DNA for transcription can be deduced from these data.

The number of binding sites for exogenous *E. coli* RNA polymerase also has impact on the interpretation of data obtained by hybridization of RNA transcribed *in vitro* from chromatin to SV40 DNA. Thus we compared the RNA syn-

TABLE I: Hybridization of SV40 DNA and RNA Synthesized *in Vitro* from Chromatin Isolated from Synchronized WIRL-3C-SV Cells at Different Stages of the Cell Cycle.[a]

RNA Synthesized *in vitro* from	Expt	cpm of RNA Syn. *in vitro* Used for Hybridzn. to 2 μg of Immobil. SV40 DNA	cpm Bound to SV40 DNA (−Background)[c]	% of Input RNA Bound to SV40 DNA
G1 chromatin	1	9×10^5	51	0.005
	2	8.8×10^5	80	0.009
Early S chromatin	1	8.7×10^5	214	0.027
	2	1.0×10^6	274	0.027
Late S chromatin	1	9×10^5	203	0.025
	2	1×10^6	208	0.020
G2 chromatin	1	9×10^5	16	0.002
	2	1×10^6	46	0.005
DNA	1	1.6×10^6	19	0.001
	2	4.7×10^6	87	0.002
Asynchronous chromatin	1	1.1×10^6	60	0.005
	2[b]	18.2×10^6	949	0.005

[a] Synchronization of cells, isolation of chromatin, and RNA synthesis *in vitro* were performed as described in Figure 3A with the exception that the volume of the reaction mixture was 10 ml, the total amount of DNA or DNA equivalents of chromatin was 850 μg, and the concentration of RNA polymerase was 0.7 mg. The reaction was allowed to proceed at 36° for 90 min and was terminated by addition of EDTA to a final concentration of 0.02 M. RNA extraction as well as its hybridization to SV40 DNA are described in Figure 2. [b] Concentration of DNA equivalents, 12 mg. [c] Background, 22–53 cpm.

thesis from constant amounts of chromatin (measured as DNA equivalents) isolated at different stages of the cell cycle with increasing concentrations of *E. coli* RNA polymerase. The chromatin concentration was chosen to be the rate limiting factor in the transcription reaction *in vitro* at the higher concentrations of the polymerase. The concentration of polymerase at which the RNA synthesis reaches a constant level is a measure of free binding sites. Figure 3B presents the results of such an experiment. Interphase chromatin is isolated at different stages of the cell cycle and 1.4 μg of DNA equivalents is transcribed with 5, 15, or 30 μg of RNA polymerase (specific activity, 2.4 units/μg of protein), respectively. The template availability of chromatin does not vary significantly throughout the cell cycle, although a marked difference between chromatin and DNA can be seen. We concluded from these data that eventual fluctuations in the synthesis *in vitro* of SV40 RNA from interphase chromatins must not be interpreted by general variations in *E. coli* RNA polymerase binding.

SV40-Specific Sequences Are Synthesized in Vitro from S-Phase Chromatin. When WIRL-3C-SV are growing randomly they contain 0.013% SV40-specific sequences in pulse-labeled RNA and 0.017% when growing synchronously during the S-phase. When WIRL-3C-SV DNA was used as a template for transcription *in vitro* and the reaction product was then hybridized to SV40 DNA, no significant hybridization was detected. However, it appeared worthwhile to determine if isolated chromatin has preserved structural features of intact cells by having covered transcription-passive DNA regions. This might allow us to detect SV40-specific sequences in the transcript *in vitro* from chromatin and also reflect the cell cycle specificity found *in vivo*.

Using conditions as described in Table I, chromatin from different stages of synchronized WIRL-3C-SV cells was used as a template to synthesize *in vitro* RNA which was purified and hybridized to excess of SV40 DNA. The transcribed RNA both from early and late S-phase chromatin shows sequences complementary to SV40 DNA. Amounts of 0.020–0.027% of total RNA synthesized were found to be SV40 specific during this stage, whereas chromatin from G1 and G2 as well as chromatin from asynchronous cells gave a transcription with a lower content in virus-specific sequences (0.002–0.009%).

Discussion

The experiments in this report are germane to two problems associated with the transcription of viral genes in transformed cells: one, the relationship between the transcription of the SV40 genome and the cell cycle, and two, the possibility of a hypothetical control exerted by the structure of chromatin on the accessibility of DNA for transcription.

It was of interest to know whether the physical association of the SV40 DNA with cellular DNA is simultaneously the basis for a functional association with the host cell. Such a functional integration could be expressed in the form of a cell cycle-dependent transcription of the integrated genome. It is well established that the replicative cycle of certain viruses is dependent on the state of the cell cycle although some of these viruses have strictly cytoplasmic replication sites (Tennant and Hand, 1970; Lawrence, 1971; Eremenko *et al.*, 1972; Leong *et al.*, 1972; Humphries and Temin, 1972), whereas the replication of other viruses has been shown to be independent of the proliferative state of the infected cell (Groyon and Kniazeff, 1967; Hodge and Scharff, 1969; Cohen *et al.*, 1971). An important advantage of the SV40 system for hybridization studies is that the viral DNA can be extensively purified and be used as a well-defined probe with simple hybridization kinetics. Using the experimental conditions described, we did not observe any release of SV40 DNA during incubation when ^{32}P-labeled SV40 DNA was immobilized on Millipore filters. An interpretation of SV40 transcription occurring specifically during the S-phase in exponentially growing cells has to be done cautiously and several possibilities can be discussed. (1) This result may be limited to the cell line used having the SV40

DNA integrated at a certain location which is transcribed during the S-phase. (2) Transcription of integrated SV40 DNA may occur only from newly replicated DNA and may be dependent on DNA synthesis. (3) The SV40 transcription in the transformed cells may be controlled as histone mRNA synthesis (Borun *et al.*, 1967; Gallwitz and Mueller, 1969; Breindl and Gallwitz, 1973), which takes place during the S-phase and is inhibited by hydroxyurea, is controlled. (4) The level of cAMP varies strongly throughout the cell cycle (Burger *et al.*, 1972) and may influence transcription.

The structure of chromatin was found not only to be organ-specific (Chytil and Spelsberg, 1971; Paul *et al.*, 1970) by antigenic and functional properties but also to vary throughout the cell cycle in one specific cell type. Sensitivity of chromatin from synchronized Hela cells to DNase I is highest during the S-phase (Pederson, 1972) and binding of actinomycin D also reaches a maximum at that stage. Alterations in the degree of chromosomal coiling extend over the entire cell cycle and may be important for transcription accessibility (Mirsky and Silverman, 1972). These fluctuations of structural composition of chromatin are paralleled by such cell cycle dependent modifications of chromosomal proteins as phosphorylation or methylation or by the quantitative amount of nonhistone chromosomal proteins (Ord and Stocken, 1968; Shepherd *et al.*, 1971; Borun *et al.*, 1972). During reaction *in vitro* of chromatin with an exogenous RNA polymerase, the transcription is qualitatively limited to a restricted set of DNA sequences giving a product which, in its hybridization characteristics, is claimed to be similar to the natural RNA synthesized from these cells (Paul *et al.*, 1970). It appeared necessary in this study before investigating chromatin from synchronous cells for SV40 specific RNA synthesis *in vitro* to quantitatively compare the binding sites for RNA polymerase in chromatin samples from different stages of the cell cycle. The fact that no major differences were detected made it easier to quantitatively relate the hybridization results. The stage in the cell cycle at which synthesis of this singular transcription product occurs is the same *in vivo* and *in vitro* suggesting an agreement with the concept that chromatin transcription may be used as a transcription *in vitro* model. Furthermore, these results strongly suggest that transcription specificity has a basis in the structural properties of chromatin.

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