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Transcription of the viral genome in cell lines transformed by simian virus 40. I. Mapping of virus-specific nuclear RNAs

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ABSTRACT

Mapping of virus-specific nuclear transcripts was carried out in three lines of rat cells transformed by SV40. Each of these cell lines contained a single copy of integrated viral DNA with identified regions adjacent to cell DNA (1). The main virus-specific nuclear transcript in all of these cell lines was shown to be complementary to the minus strand of the early region in SV40 genome. Each cell nucleus contained approximately 50 copies of these RNAs. Transcripts complementary to both strands of the late region in viral genome were also detectable in all of these cell lines. Its content varied depending on the cell line and was 20-50-fold less than that of the main virus-specific transcript. All the regions of integrated SV40 genome in isolated nuclei of transformed cells were equally sensitive to pancreatic DNase I treatment suggesting that the whole viral genome served as a template for RNA synthesis in these cell lines.

INTRODUCTION

Transformation of nonpermissive rodent cells by Simian Virus 40 (SV40) is accompanied by covalent integration of viral DNA into the host-cell genome (2) and its continued expression (3,4,5). The results of studies on the transcription of SV40 genome in several transformed cell lines indicate that virus-specific RNA is similar but not identical to that found at the early stage of productive infection. The main virus-specific RNA in the transformed cells is 19 S RNA (6). It is complementary to the minus strand of the early region of SV40 genome (7) and seems to be identical to the early mRNA from productively infected monkey cells. There are also variable amounts of anti-late (8,9,10) and even late (10,11) RNAs. Under the conditions of pulse labeling, long viral transcripts were observed that exceede length the size of the SV40 DNA strand (12) and may con-

tain viral and host-cell sequences in the same molecule (13). It has been suggested that these species of RNA can serve as precursors of 19S mRNA and that its synthesis may be initiated on host-cell promoters.

Strict interpretation of the data concerning viral transcription in transformed cells is difficult owing to the absence of information about the state of SV40 genome in the cell lines used in these studies. It is known that many cell lines contain multiple copies of integrated viral DNA. Recent studies have revealed that significant perturbations may occur in the course of integration. As a result of these perturbations, different regions of SV40 genome may be present in a nonequal number of copies (14) and organized in tandems (1). It is apparent that transcription on such altered templates can yield high molecular weight RNA as well as transcripts from the late region of SV40 genome. This may particulary be the result of the loss of regions of the viral genome which are responsible for RNA chain termination or that of the regions carrying sequences which are recognized by processing enzymes. It is also possible that some of these transcripts are functionally inactive and do not serve as precursors of mature mRNAs.

The present report is concerned with the study of virus-specific transcription in three cloned SV40 transformed rat cell lines which contain only one copy of the entire SV40 DNA. The state of viral genome in these cell lines has been characterized in detail by Botchan et al. (1). Different regions of SV40 genome in these cell lines are adjacent to cellular DNA and viral DNA insertion contains an unbroken early region surrounded by parts of the late region of SV40 DNA. It was found that though tge main virus-specific transcript in all of these cell lines is early RNA, late and anti-late RNAs mapping around the region of SV40 genome coding early mRNA are also detectable.

MATERIALS AND METHODS

Cell cultures. Cloned SV40-transformed cells (clones 12, 14B and 17, see ref.1,5) were grown in roller bottles in the Minimal Essential Medium supplemented with 10% calf serum and 200 μg/ml of kanamycin. A continous line of African green monkey

cells (CV1) was grown under similar conditions.

SV40 DNA. SV40 DNA was isolated by the Hirt selective extraction procedure (16) and was further purified in two cycles of CsCl -ethidium bromide buoyant density centrifugation.

Preparation of specific fragments of SV40 DNA. Three specific fragments of SV40 DNA produced by cleavage with restriction endonucleases Eco RI, Bam HI and Bgl-I were used as probes for hybridization and reassociation in these studies. The large fragment A (0.15-0.67 map units) included the whole early region of SV40 genome (0.17-0.67) and a small part of the late region (0.15-0.17). Two other fragments B (0.67-1.00) and C (0.00-0.17) represented almost the entire late region.

100 µg of SV40 DNA was treated with 100 units of each of Eco RI, Bam HI and Bgl-I restriction endonucleases in a volume of 200 µl of 0.01 M tris-HCl, pH 7.6, 0.01 M $MgCl_2$, 0.001 M dithiotreitol for 3 hours at 37°C. The reaction was terminated by adding 0.005 M EDTA, and DNA fragments were separated by electrophoresis in 1.4% agarose gels as described (18) in a slab gel apparatus (19). The gels were stained with 0.5 µg/ml of ethidium bromide, and DNA bands were visualized under ultraviolet light. DNA was extracted from the gel by the freezing and thawing procedure and recovered by ethanol precipitation. It was then dissolved in a small volume of 0.001 M NaCl and used for enzymatic labeling in vitro.

Preparation of radioactive DNA probe. SV40 DNA and specific fragments of DNA were labeled in vitro to a high specific radioactivity by nick-translation (20). The reaction was carried out in a volume of 25 µl of a mixture containing 0.1 µg of DNA, 2 units of DNA polymerase I (E.coli, Boehringer/Mannheim, Grade 1), 2 µl of each of α-^{32}P-deoxyribonucleoside triphosphates (Amersham, England, 100-250 Ci/mM), 0.05 M tris-HCl, pH 7.8, 0.005 M $MgCl_2$, 0.005 M β-mercaptoethanol, for 60 minutes at 15°C. The reaction was terminated with 0.05 M EDTA and 0.5% SDS, DNA was extracted twice with phenol-chloroform -isoamyl alcohol and passed through a Sephadex G-50 column. The specific radioactivity of DNA preparations was within a range of $5 \times 10^7 - 2 \times 10^8$ cpm/µg. DNA was fragmented to a size of approximately 300 nucleotides by boiling for 20 minutes in 0.3 N NaOH. To get rid of rapidly annealing

hairpin DNA structures that may arise in the course of nick-translation, DNA preparations were passed through a hydroxyapatite column in 0.14 M Na-phosphate, pH 6.8, at 60°C. DNA strand separation was performed as described (21). 50 ng of labeled denatured fragmented DNA was annealed for 3 hours in a volume of 200 ul of 0.3 M NaCl, 0.14 M Na-phosphate, pH 6.8, 0.4% SDS with 5 µg of self-annealed asymmetric complementary RNA synthesized in vitro by E.coli RNA polymerase (22). The hybridization mixture was then diluted 5-fold with water and applied on a 0.2 ml hydroxyapatite column at 60°C. Single-stranded DNA was eluted with 5 column volumes of 0.125 M Na-phosphate, pH 6.8, 0.4% SDS, while RNA-DNA hybrid molecules were eluted with 5 column volumes of 0.25 M Na-phosphate, pH 6.8, 0.4% SDS. RNA was eliminated by incubation in 0.2 N NaOH for 90 min at 45°C, and DNA was self-annealed: 20 ng of DNA was incubated in 500 µl of 1 M NaCl, 0.14 M Na-phosphate, pH 6.8, 0.4% SDS, for 16 hours at 65°C. Single-stranded DNA was recovered by hydroxyapatite chromatography and was considered to represent separated strands of viral DNA. Only minus DNA strand preparations were shown to be able of forming duplex molecules with cRNA preparations as revealed by hydroxyapatite chromatography.

Treatment of transformed cell nuclei with DNase I and isolation of cellular DNA. Transformed cells were grown in a medium containing 2 µCi/ml of ^{3}H-thymidine (10 Ci/mM, 'Isotop', USSR). The cells were removed from the glass surface with a 0.02% Versene solution and washed three times with isotonic Na-phosphate buffer, pH 7.3, containing 10 mM $MgCl_2$. After that, the cells were washed with RSB (0.01 M NaCl, 0.01 M tris-HCl, pH 7.4, 0.003 M $MgCl_2$) and lysed with 0.5% Nonidet P40. The nuclei were suspended in RSB at a DNA concentration of 1 mg/ml and digested as described (23). The reaction was terminated by placing the reaction vial on ice and adding 0.005 M EDTA. The nuclei were pelleted by low speed centrifugation, suspended in 0.005 M EDTA, and treated with 0.5 mg/ml of pronase (Calbiochem) in 0.5% SDS for 6 hours at 37°C. Nucleic acids were extracted with phenol-chloroform -isoamyl alcohol, and RNA was eliminated by incubation in 0.2 N NaOH for 5 hours at 45°C. The solution was neutralized and DNA was recovered by ethanol precipitation. DNA prepa-

rations from DNase I digested nuclei were analyzed on 3% alcaline agarose gels (24) with Hae-III digest of SV40 DNA as a marker. Most of the DNA had a size of approximately 150 nucleotides. DNA from the nuclei nontreated with DNase I was isolated by the same method. Then, it was fragmented to a size of approximately 150 nucleotides by boiling in 0.3 N NaOH for 40 min.

Isolation of nuclear RNA. The nuclei of transformed cells were isolated according to Penman (25). The cells were swollen in RSB for 10 min on ice and disrupted in a Dounce homogenizator. The nuclei were pelleted by low speed centrifugation, treated with 0.5% deoxycholate-Na - 1% Tween 40 in RSB and collected by centrifugation. DNA was extracted with hot phenol as was described (26), treated with DNase I and passed through Sephadex G-75 column. 5 mg of nuclear RNA was routinely prepared from 1 x 10^9 cells. No mature ribosomal RNA species were observed in nuclear RNA preparations indicating the absence of significant contamination with cytoplasmic RNA.

DNA-RNA hybridization. DNA-RNA hybridization was carried out in a mixture containing 1 M NaCl, 0.05 M Na-phosphate, pH 6.8, 0.1% SDS, 1 x 10^{-3} µg/ml of labeled plus and minus DNA strands, and 0.1 - 10 mg/ml of RNA from the transformed cell nuclei at 65°C for 40 hours. The mixture was then diluted 10-fold with 0.125 M Na-phosphate, pH 6.8, 0.4% SDS, and the fraction of RNA-DNA hybrid molecules was determined by chromatography at 65°C (27).

DNA-DNA reassociation. 2.4 x 10^{-4} µg/ml of denatured labeled viral DNA was annealed in the presence of 1.5 mg/ml of denatured fragmented DNA from transformed cells in 1 M NaCl, 0.14 M Na-phosphate, pH 6.8, 0.2% SDS at 65°C under mineral oil (Bayol F, Serva). Aliquots were taken at intervals and the fraction of double-stranded DNA was analyzed by hydroxyapatite chromatography (21). The number of copies of viral DNA in the host-cell genome was calculated as described (28).

Enzymes. Restriction endonucleases Bam HI and Bgl-I were isolated as described (29,30); Eco RI which was isolated by the method of Green et al. (31) was kindly donated by Dr. S.Nedospasov. E.coli RNA polymerase was a gift from Dr. A.Gragerov. DNA polymerase I of E.coli was purchased from Boehringer/Mannheim. DNase I, free of RNase, was obtained from Worthington.

RESULTS AND DISCUSSION

Analysis of transformed cell nuclear RNA derived from different regions of SV40 genome. Separated strands of labeled SV40 DNA fragments were annealed with different concentrations of RNA extracted from the nuclei of transformed cell lines. The amount of the labeled DNA probe that entered hybrid molecules was determined by chromatography on hydroxyapatite. In control experiment, the labeled DNA probe was annealed with nuclear RNA from nontransformed cells and with cRNA synthesized *in vitro* on SV40 DNA by *E.coli* RNA polymerase. Less than 5% of the DNA probe was retained on a hydroxyapatite column after elution of single-stranded DNA when it was annealed with control mouse cell nuclear RNA, whereas more than 85% of the minus strand DNA was eluted as a RNA-DNA duplex after annealing with an excess of SV40 cRNA. Since cRNA is complementary only to the minus strand of SV40 DNA, the ability of the plus DNA strand preparations to serve as a hybridization probe was checked by the other method, namely, by testing its ability to reassociate in the presence of an excess of unlabeled denatured SV40 DNA. According to this test, the preparations of the plus strand were identical to those of the minus strand.

The extent of hybridization of different fragments from SV40 genome with nuclear RNA from transformed cells is shown on Fig.1. In all of the cell lines tested, the main virus-specific transcript corresponded to the minus strand of the region encompassed by fragment A. The DNA probe of this fragment was saturated at 1 - 2.5 mg of nuclear RNA per 1 ml, and virtually all DNA entered the hybrid at this RNA concentration. In contrast, the plus strand of the same fragment A did not actually react with RNA. Two other fragments, B and C, did hybridize with nuclear RNA but the concentrations of RNA corresponding of these fragments were very low and, within the range of nuclear RNA concentrations used in these experiments, it was impossible to reach the saturation of the DNA probe. It has not been possible therefore to establish from this experiment, which part of the sequences of these fragments is present in RNA transcripts.

Transcripts from both strands of fragments B and C were found in nuclear RNA, but the relative amounts of these trans-

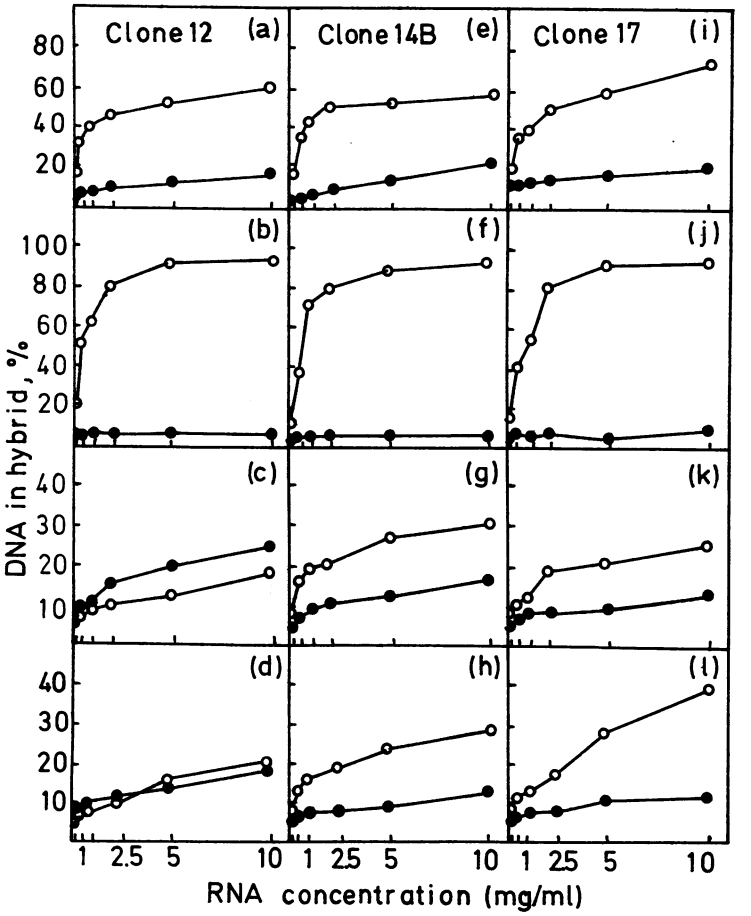

Fig.1. Kinetics of hybridization of separated minus (o) and plus (o) strands of SV40 DNA fragments produced by Eco RI, Bam HI and Bgl-I restriction endonucleases with different amounts of RNA extracted from the nuclei of three SV40 transformed cell lines. The conditions of hybridization were as described in Matherials and Methods. Each point of the curve is a mean value from three independent experiments. (a),(e),(i) - hybridization with separated strands of entire SV40 DNA; (b), (f),(j) - with fragment A; (c),(g),(k) - with fragment B; (d), (h),(l) - with fragment C.

cripts varied among different cell lines. The largest amount of plus strand transcripts was observed in clone 12. No transcripts corresponding to the plus strand of fragment A were observed in

this experiment.

For quantitative estimation of transcripts from different regions of SV40 genome the hybridization curves shown in Fig.1 were compared with those obtained in the reconstruction experiment when the minus strands of DNA fragments were hybridized with control mouse cell nuclear RNA containing various concentrations of SV40 cRNA (see Fig.2). It can be seen that the hybridization curve for the minus strand of fragment A with transformed cell nuclear RNA resembles closely that with control RNA containing 0.001% of cRNA. Similarily, it is likely that the amount of late and anti-late transcripts (complementary to fragments B and C) is close to 0.000025-0.00005%. Obviously, since it is not clear which part of the sequences of fragments B and C is present in nuclear RNA, the quantity of these transcripts can be estimated only very roughly. Nevertheless, it may be con-

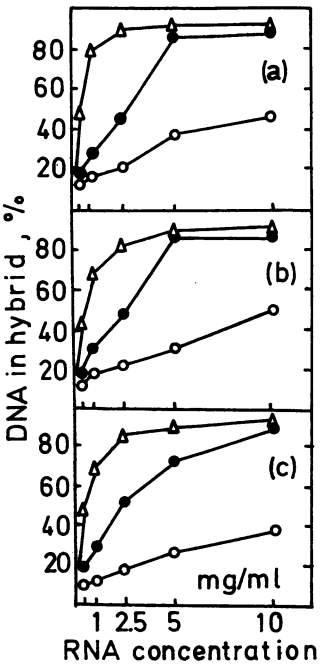

Fig.2. Hybridization of the minus DNA strand of (a) fragment A; (b) fragment B; and (c) fragment C of SV40 DNA with nontransformed cell nuclear RNA to which 0.00005% (o), 0.00025% (o) and 0.001% ( ) of asymmetric SV40 cRNA were added.

cluded that the amount of early transcripts in nuclear RNA is 20-50 times higher than that of anti-late and late RNA.

Sensitivity of viral DNA templates in isolated nuclei of transformed cells to limited digestion with pancreatic DNase I. Since the amount of late region transcripts in the nuclei of transformed cells is very low, one can consider the possibility that this is the result of unusual transcription in some of the cells, i.e. the consequence of extensive symmetrical transcription of free SV40 DNAs that might arise through occasional excision of the integrated viral genome. Since excision of viral genome from the DNA of SV40 transformed cell lines occur rarely, it is evidently that the late region of the viral genome would serve as a template for transcription only in a small number of cells. To test this possibility, a method was used that allows one to eliminate actively transcribed DNA templates. This method is based on the observations that transcribed genes in isolated nuclei are more sensitive to DNase I than the total DNA (23). The enhanced susceptibility of DNA template to DNase I does not depend upon the abundance of RNA transcripts derived from it (32). Non-transcribed regions of DNA adjacent to the transcribed ones seem to retain the resistance to DNase I (33). These data allow one to use DNase I treatment of the cell nuclei as a method for selective elimination of transcribed DNA sequiences.

In the present study, SV40 DNA sequiences were analyzed in DNA preparations isolated from DNase I digested and non-digested nuclei of transformed cells. DNase I digestion was carried out as described in Materials and Methods. The quantity of viral sequences in the DNA preparations was determined from the difference in the kinetics of reassociation of labeled SV40 DNA fragments in the presence of DNA extracted from DNase I digested and non-digested nuclei.

Since active DNA is degested more rapidly than the total DNA (23), virtually all of it can be eliminated upon some extent of digestion. To be certain that all of the active DNA templates were eliminated, DNase I treatment was performed under two different sets of conditions allowing elimination of approximately 10 and 15% of the total DNA. The conditions of digestion differed in the time of treatment at the same DNase I concentration.

The extent of digestion was monitored by measuring the part of acid-precipitable labeled DNA. The absence of significant difference in the amount of viral sequences in DNA preparations digested under both sets of conditions indicated that virtually all of active templates with enhanced susceptibility to DNase I were eliminated.

The specificity of DNase I action was tested by quantitation of viral sequences in the DNA preparations isolated from nuclei treated with staphylococcal nuclease to the similar extent of digestion. No difference was observed between the rate of reassociation of the SV40 DNA probe in the presence of DNA from non-treated and treated with staphylococcal nuclease nuclei (data not shown).

As can be seen from Table 1 and Fig.3, the DNA of the cell lines tested contained approximately one genome equivalent of SV40 DNA per diploid quantity of cellular DNA. All regions of SV40 genome corresponding to different fragments used as a probe were present in nearly equimolar amounts. This result is consistent with the observations of Botchan et al. (1) indicating that each of these cell lines contains a single copy of the entire SV40 DNA molecule. Firthermore, DNase I treatment eliminated 70-90% of SV40 DNA sequences under the conditions when only 10% of the total DNA was eliminated. No difference was observed between the sensitivities of different parts of SV40 genome to DNase I treatment in all of the cell lines. In spite of great differences in the abundance of transcripts corresponding to the early and late regions of SV40 genome, fragment A and fragments B and C were digested to the same extent. It seems therefore unlikely that transcripts from the late region of SV40 genome originate from rare nonintegrated viral molecules. However, it was impossible to eliminate all of the viral DNA sequences even at the high extent of digestion of the total cellular DNA. This fact indicates that viral sequences are not active in some of the cells. This is probably due to nonsynchronized cell populations used in these experiments. Since it is known that the expression of viral genes in transformed cells depend on the stage of cellular cycle (34), one could expect that the viral DNA insertion may be in a transcriptionaly inactive, resistant

Table 1

Reassociation of Labeled SV40 DNA and Viral DNA Fragments in the presence of Nondigested and DNase-I-Digested Nuclei of Three Transformed Cell Lines.

Clone DNA	labeled probe	$t_{1/2}$ *	number of viral DNA copies per diploid genome	percentage of viral sequences eliminated by DNase I treatment
I				
12	A	20.38 ± 5.46	1.07	--
	B	17.14 ± 4.99	1.32	--
	C	19.36 ± 5.92	1.16	--
	SV40	20.85 ± 7.47	1.15	--
14B	A	17.39 ± 4.47	1.31	--
	B	18.72 ± 3.14	1.16	--
	C	20.60 ± 3.09	1.01	--
	SV40	21.59 ± 5.18	1.10	--
17	A	21.65 ± 3.18	0.99	--
	B	20.44 ± 8.90	1.01	--
	C	18.48 ± 5.19	1.27	--
	SV40	22.83 ± 6.81	1.03	--
II.				
12	A	44.86 ± 12.8	0.32	70.09
	B	39.24 ± 6.17	0.29	78.03
(11%)**	C	32.20 ± 4.98	0.22	81.03
	SV40	68.20 ± 10.1	0.23	80.00
14B	A	42.50 ± 9.39	0.35	73.28
	B	35.52 ± 7.62	0.37	68.10
(7%)	C	31.85 ± 3.39	0.24	76.23
	SV40	55.80 ± 11.1	0.32	70.90
17	A	50.49 ± 10.5	0.24	75.70
	B	42.12 ± 7.82	0.23	77.45
(12%)	C	35.40 ± 3.12	0.09	92.90
	SV40	102.7 ± 20.2	0.10	90.30
III.				
12	A	48.12 ± 9.40	0.27	74.76
	B	40.44 ± 5.05	0.27	79.54
(15%)	C	31.82 ± 6.80	0.24	79.31
	SV40	76.46 ± 12.7	0.20	82.61
14B	A	44.79 ± 4.73	0.32	75.57
	B	41.78 ± 6.48	0.24	79.31
(18%)	C	32.79 ± 3.17	0.20	80.19
	SV40	63.33 ± 24.8	0.26	76.61
17	A	48.12 ± 8.70	0.27	72.70
	B	43.76 ± 6.08	0.21	79.40
(17%)	C	34.43 ± 3.95	0.13	89.76
	SV40	96.50 ± 15.5	0.12	88.34

* $t_{1/2}$ is time required for renaturation of a half of the DNA probe. The number of copies of viral DNA per diploid quantity of cell genome was calculated as described (28).

** the percentage of the total DNA eliminated by DNase I treatment.

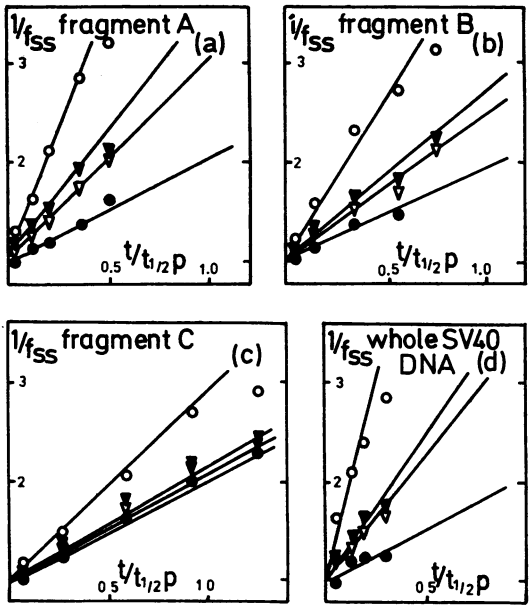

Fig.3. Kinetics of reassociation of ^{32}P-labeled DNA of fragment A (a), fragment B (b), fragment C (c) and of the whole SV40 DNA (d) in the presence of DNA extracted from transformed cells (clone 14B) - (o) and in the presence of DNA extracted from the nuclei of the same cell line treated with DNase I under the conditions when 7% ( ) and 15% ( ) of the total DNA become acid-soluble. Reassociations were carried out as described in Materials and Methods. The specific activities of the DNA probe were 1.1 x 10^8 cpm/ug for fragment A; 1.25 x 10^8 cpm/ug for fragment B; 0.98 x 10^8 cpm/ug for fragment Cand 1.3 x 10^8 cpm/ug for the whole SV40 DNA. The concentration of labeled DNA probe in each reaction mixture was 2.5 x 10^{-4} ug/ml. The time required to reassociate a half of the DNA probe in the presence of control calf thymus DNA ($t_{1/2}p$) was 90.11 ± 9.45 hours for fragment A; 61.85 ± 5.99 hours for fragment B; 38.22 ± 2.46 hours for fragment C and 164.28 ± 34.68 hours for the whole SV40 DNA. (o) - reassociation of labeled DNA in the presence of control calf thymus DNA.

to DNase I conformation in some of the cells.

The data presented above indicate that not only early region of SV40 genome necessary for cell transformation was transcribed in three lines of transformed cells containing a single integrated viral DNA. Late and anti-late nuclear transcripts mapping around the early region of SV40 genome were also

detectable although in a much lower amount. Since it was impossible to detect in these experiments what part of the sequences of the late region was present in RNA transcripts, the origin of these transcripts is not clear enough. It is evident from the known points of integration in the viral genome (1) that not all of the late transcripts may initiate on the late lytic promoter mapping near the 5′end of the late mRNA, It is possible that some of the plus strand transcripts observed in these studies may initiate on host promoters, but we have no additional data supporting this possibility. Recent studies from this laboratory revealed that not all of the poly(A) stretches in more than genome length viral nuclear transcripts isolated from these cells were protected from RNase A cleavage after annealing with the DNA of the early region (fragment A) suggesting that poly(A) in some of the virus-specific RNA is separated from the early sequences by either anti-late, or host RNA sequences (35). It is likely that at least some of the RNA from the minus strand of SV40 genome fail to terminate in the end of the early region resulting in the appearence of anti-late RNA mapping downstream from the early mRNA. Firthermore, it seems likely that not all of the minus-strand viral transcripts were initiated on the promoter mapping just upstream from the early region of SV40 genome, but some of the RNA may initiate either on the viral promoter(s) located in the late region of SV40 genome, or on the cellular promoters. It has been shown recently the proportions of nuclear transcripts from different regions of SV40 genome under long steady state and very short pulse labeling were very similar (36). Therefore, it is probable that the major part of SV40 mRNA is initiated on the viral promoter mapping near 5′ end of the early mRNA. More information must be obtained to reveal the significance of non-early nuclear transcripts.

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