1. INTRODUCTION

Expression of the SV-40 genome in transformed mouse cells (ST3) and in lytically infected monkey cells (BSC-1) seems to be regulated. In SV-3T3 cells, the viral genes are expressed only partially; a virus-specific nuclear antigen (V antigen) (Black et al., 1963) is detectable but neither autonomous replication of viral DNA nor synthesis of capsid protein (V antigen) takes place. In BSC-1 cells infected by SV-40, V antigen appears soon after infection, independent of viral DNA replication ("early" time) (Sabin and Koch, 1964; Hopp et al., 1964) while V antigen is detectable only after viral DNA replication starts ("late" time) (Gerstein and Sachs, 1964).

By RNA-DNA hybridization competition experiments, both Oda and Dulbecco (Oda and Dulbecco, 1968) and Aloni (Aloni et al., 1968) showed that SV-40 RNA from SV-3T3 cells lacks 60% to 70% of the base sequences of the viral RNA synthesized at a "late" time after lytic infection of BSC-1 cells. Since the whole viral genome persists in the SV-3T3 cells (Watkins and Dulbecco, 1967), their results suggested that the expression of the viral gene is controlled at the transcriptional level. Their results, however, did not exclude the possibility that the whole viral genome is transcribed once and viral RNA with certain base sequences (such as those in the capsid protein gene) is selectively degraded later.
The recent finding by Lindberg and Darnell of large viral RNA in the nuclei of SV 373 cells is consistent with this notion (Lindberg and Darnell, 1970). A similar argument applies also to "early" viral RNA.

In order to obtain basic information on the regulation of viral gene expression we have been characterizing viral RNA in SV 373 cells, as well as in BSC-1 cells lytically infected by SV 40.

2. GENERAL METHODS

SV 373 cells were grown at 37°C on 8 cm Nunc plastic petri dishes in reinforced Eagle's medium containing 10% fetal serum. BSC-1 cells were grown similarly with 10% fetal calf serum and 10% tryptose phosphate and infected with SV 40 at a m.o.i. of 50 to 100.

RNA was labeled with 200 to 500 μCi per petri dish of 32P-uridine (500 Ci/m mole) in the presence of 2% dialyzed serum. Extraction of RNA was carried out at 0-4°C by a modification of the method of Kirby (Kirby, 1968).

Separation of nuclei and cytoplasm was performed by the Penman procedure (Penman, 1966).

DMSO gradient centrifugation was undertaken as described by Strauss et al. (Strauss et al., 1968).

RNA-DNA hybridization and RNA-DNA hybridization competition experiments were carried out according to Westphal and Dulbecco (Westphal and Dulbecco, 1968) and Tonegawa and Hayashi (Tonegawa and Hayashi, 1968) respectively.

Polyacrylamide gel electrophoresis was performed by the method of Loening (Loening, 1967) and Summers (Summers, 1969). The gel concentration was 2.2% with 0.5% acrylamide. The gel was fractionated by use of a Savant gel slice. In order to determine the M, of the RNA, determined by polyacrylamide gel electrophoresis to that determined by DMSO gradient, in vitro synthesized SV 40 RNA (Westphal and Kusm, 1970) with various selected sizes were subjected both to DMSO gradient and polyacrylamide gel electrophoresis with ribosomal RNA's as the standards.

The molecular weights of viral RNA as determined by gel electrophoresis were 1.43 ± 0.02 times higher than that determined by DMSO gradient (Tonegawa et al., 1970).

3. RESULTS

Fig. 1a shows the sedimentation profiles of SV 373 RNA which was labeled with 32P-uridine for 60 min. RNA hybridizable with SV 40 DNA is distributed in two rather broad bands. These viral RNA components are hereafter called "large" viral RNA. A shoulder is also observed under 18S ribosomal RNA marker. This viral RNA component is hereafter called "small" viral RNA.

Strauss et al. (Strauss et al., 1968) showed that the secondary structure of bacteriaeose RNA and a polymer, rGorC, are completely destroyed in 95% diethylpyrocarbonate (DMO) at room temperature, thus under the sedimentation conditions used in the present work, all species of RNA are expected to sediment as a function of the chain length. Ring-shaped SV 40 DNA with a single nick in one of the two strands sedimented closely together with the 28S ribosomal RNA marker under the conditions used (data not shown). Thus in fig. 1a approximately 26% of the RNA hybridizable with SV 40 DNA are not smaller than a single strand of SV 40 DNA.
The specificity of the hybridization was checked by use of RNA from 3T3 cells in untransformed mouse cell line. This RNA, when sedimented in DMSO, cross-hybridized with SV40 DNA at a level no more than 0.001% of the input as the way through the gradient (data not shown). The counts in the hybrids at the two peak positions in fig. 1a were 0.0006% and 0.0033% of the respective inputs.

In order to study the precursor-product relationship between the "large" viral RNA's and the "small" viral RNA, "pulse-chase" experiments were carried out. Fig. 1b shows the sedimentation profiles of SV3T3 RNA chased with unlabeled uridine for six hours after one hour pulse with 3H-uridine. Counts in the "small" viral RNA are predominant in contrast to fig. 1a. The residual incorporation of 3H-uridine during the chase periods and possible reutilization of nucleotides (Baltimore, 1970) make the quantitative analysis of this experiment rather difficult.

In order to circumvent these difficulties, a similar pulse-chase experiment was carried out with 10 μg/ml of actinomycin D during the chase periods and results are shown in fig. 2. Since shut-off of RNA synthesis is near complete under this condition, the increase of the counts in the "small" viral RNA during the chase periods must be attributed to conversion from the "large" viral RNA.

From these experiments, we conclude that the "large" viral RNA are precursors of the "small" viral RNA.
Fig. 2. Pulse-chase experiment of SVT3 cells with actinomycin D during chase. --- total RNA; no total viral RNA; ■ "large viral RNA"; ○ "small viral RNA". Results are expressed as cpm/100 µg of total RNA.

The intracellular distribution of the viral RNA components was examined by analyzing the RNA prepared from isolated nuclei or cytoplasm of SVT3 cells. The results show that the "large" viral RNA and the "small" viral RNA are richer in the nuclei (Fig. 3, top) and cytoplasm (Fig. 3, bottom), respectively.

Fig. 3. Distribution in DAB gradient of viral RNA in the nucleus and cytoplasm of SVT3 cells. Labeling time was 90 min. Under the conditions used, the purified RNA, as calculated from the optical density at 260 mg/ml, was distributed between the nuclear and cytoplasmic fractions at a ratio of 1:6.18. The amount of RNA layered in 24 µg of nuclear RNA, 60 µg of cytoplasmic RNA is layered.
The intracellular distribution of the viral RNA components was examined by analyzing the RNA prepared from isolated nuclei or cytoplasm of SV3T3 cells. The results show that the "large" viral RNA and the "small" viral RNA are richer in the nuclei (Fig. 3, top) and cytoplasm (Fig. 3, bottom), respectively.
The "small" viral RNA in the nucleus preparation may be at least partially explained by contamination from the cytoplasm because the relative amounts of the 43S ribosomal precursor RNA and 18S ribosomal RNA indicate that the nucleus preparation is only 75% to 80% pure. On the other hand, the near complete absence of the 43S and 32S ribosomal RNA precursors indicates that the cytoplasmic fraction has a high degree of purity. In spite of this, there seems to be also a small amount of "large" viral RNA in the cytoplasm. These results suggest that at least a part of the conversion process takes place in the cytoplasm. It is noteworthy that no "large" viral RNA sedimenting faster than 28S ribosomal RNA is found in the cytoplasm.

As discussed in the introduction, previous studies by use of RNA-DNA hybridization suggested that the regulation of viral gene expression in SV 3T3 cells is on the transcriptional level. The finding of the "large" precursor viral RNA's with a shorter half-life, however, opened the possibility that the whole viral genome is transcribed but specific sections of the RNA molecules are degraded later.

In order to differentiate between these models, the "large" precursor viral RNA was examined to see whether it contains "late" sequences. In one experiment, either pulsed or pulse-chased total SV 3T3 RNA was competed with unlabeled RNA isolated from SV 3T3 for sites on the SV 40 DNA. The pulsed RNA sample consisted of 80% of "large" viral RNA and 20% of "small" viral RNA, whereas the chased RNA sample consisted of 15% of "large" viral RNA and 85% of "small" viral RNA. Fig. 4 shows the results of the competition experiment.

Fig. 4. Competition of pulsed or pulse-chased SV 3T3 RNA with total labeled SV 3T3 RNA. The pulse was for one hour and the chase was for 6.5 hours. The indicated amount of unlabeled SV 3T3 RNA was first hybridized with 0.1 μg of 3H-labeled SV 40 DNA for 16-18 hours; then the labeled RNA was added and the mixture was incubated for an additional 16 hours at 0°C. The filled circles and the open circles represent two different experiments. - - - - - represents the experiment in which unlabeled RNA isolated from secondary mouse embryo (S.E.) cells was used as the competitor.
Both pulse and pulse-chased RNA were completely competed by unlabeled SV 3T3 RNA. Furthermore, there were essentially no differences between the two cases in the competing efficiency of unlabeled SV 3T3 RNA. Unlabeled RNA isolated from the secondary culture of mouse embryo cells competed only 1% indicating that the observed total competition with SV 3T3 RNA is due to viral RNA.

In another experiment, the gradient fractions containing the "large" viral RNA were pooled and used in the competition with unlabeled SV 3T3 RNA (fig. 5). The results again show that at least 90% of the base sequences in the "large" viral RNA's are competed by unlabeled SV 3T3 RNA. The outcome of the competition experiment between "late" lytic RNA and SV 3T3 RNA indicates that the unlabeled SV 3T3 RNA used in these experiments lacks "late" base sequences in conformations with previous results by others (Osh and Dulbecco, 1968; Amon et al., 1968). From these experiments, we conclude that the "large" viral RNA contains no appreciable amount of "late" viral RNA base sequences.

In order to resolve the multicomponent of the "large" precursor RNA, SV 3T3 RNA was subjected to polyacrylamide gel electrophoresis. In comparing the molecular weight of in vitro transcribed SV 40 RNA determined by DMSO gradients and polyacrylamide gel electrophoresis, it was found that the apparent molecular weight in polyacrylamide gel was 1.4 times larger than that in DMSO gradient, using ribosomal RNA as the marker (Tomogawa et al., 1970). Based on the assumption that in DMSO all species of RNA molecules assume a random configuration, we can conclude that under the conditions of gel electrophoresis ribosomal RNA and SV 40 RNA differ in their secondary structures. Therefore, molecular weights of viral RNA determined by acrylamide gel electrophoresis using ribosomal RNA markers were corrected accordingly.
Fig. 6. Polyacrylamide gel electrophoresograms of pulsed or pulse-chased SV3T3 RNA. RNA was from the line gel bands by freezing and thawing, and then hybridized with 3.6 μg SV40 DNA. (a) one hour pulse; (b) four hour chase after one hour pulse. This continuous line represents ribosomal RNA.

Fig. 6 shows gel electrophoresograms of RNA prepared from SV3T3 cells pulse-labeled for one hour with $^{3}H$-uridine (fig. 6a) and chased for four hours after a one-hour pulse (fig. 6b). The molecular weight of the fastest-migrating peak (fraction 29 of fig. 6a or fraction 31 of fig. 6b) was estimated as $8.5 \times 10^{6}$ daltons which is approximately 50% of the molecular weight of a single strand of SV40 DNA. The "large" viral RNA now seems to be resolved to multicomponents whose molecular range was $1.25 \times 3 \times 10^{6}$ daltons. There also is an even larger component which did not migrate into the gel. Consistent with the pulse-chase experiments in the DMSO gradient (fig. 1), the counts were reduced in these "large" viral RNA's upon chase and accumulated in the $8.5 \times 10^{6}$ dalton species. Westphal and Dulbecco (Westphal and Dulbecco, 1968) found that the SV3T3 cells contain 20 genome equivalents of SV40 DNA per cell. The multiple precursor RNA species may arise from different integration sites, each with a different contribution of cellular RNA. (See Discussion).
Fig. 6. Polyacrylamide gel electrophoretograms of pulsed or pulse-chased SV 3T3 RNA. RNA was obtained from the live cell lines by freezing and thawing, and then hybridized with 2.5 µg SV 40 DNA. a: one hour pulse; b: four hour chase after one hour pulse. Thin continuous line represents ribosomal RNA.

Fig. 6 shows gel electrophoretograms of RNA prepared from SV 3T3 cells pulse-labeled for one hour with 3H-uridine (Fig. 6a) and chased for four hours after a one-hour pulse (Fig. 6b). The molecular weight of the fastest-migrating peak (fraction 28 of fig. 6a or fraction 31 of fig. 6b) was estimated as 8.5 x 10^6 daltons which is approximately 50% of the molecular weight of a single strand of SV 40 DNA. The "large" viral RNA now seems to be resolved into multicomponents whose molecular range was 1.25 to 3 x 10^6 daltons. There is also an even larger component which did not migrate into the gel. Consistent with the pulse-chase experiments in the DMIBS gradient (Fig. 1), the counts were reduced in these "large" viral RNA's upon chase and accumulated in the 8.5 x 10^5 dalton species. Westphal and Delbacco (Westphal and Delbacco, 1966) found that the SV 3T3 cells contain 20 genome equivalents of SV 40 DNA per cell. The multiple precursor RNA species may arise from different integration sites, each with a different contribution of cellular RNA. (See Discussion).
The sedimentation profiles of RNA prepared from uninfected and SV 40-infected BSC-1 cells are shown in Fig. 7. In Fig. 7b, RNA was labeled with $^{32}P$-uridine from 10 to 11 hours after infection and 5 x $10^{-5}$ M 5-Fluorodeoxyuridine (FUDR) was present in the medium from 0 to 11 hours after infection to prevent viral DNA replication; this is "early" RNA. The counts hybridizable with SV 40 DNA were $0.02\%$. The main peak of RNA hybridizable with SV 40 DNA sedimented together with 18S ribosomal RNA marker and therefore its molecular weight is approximately $6.5 \times 10^6$ daltons. There is also a shoulder on the faster side of this main peak and a minor peak on the slower side.

The approximate molecular weight of the latter is $2.5 \times 10^6$ daltons. Approximately one-third of the total hybridizable counts sediment very fast. The estimated molecular weight of the fraction of RNA ranges between $2 \times 10^6$ and $6 \times 10^6$ daltons, corresponding in size to more than one genome length. The sedimentation profile of hybridizable RNA from cells unexposed to FUDR and labeled from 10 to 11 hours after infection was essentially the same as in Fig. 7b (data not shown).

When SV 40-infected cells were labeled with $^{32}P$-uridine (Fig. 7c) from 40 to 50 hours after infection, 5% of the $^{32}P$ counts in the extracted RNA hybridize with SV 40 DNA; this is the "late" RNA. The position of the peak of viral RNA again corresponds approximately to $6.3 \times 10^6$ daltons. A significant fraction of the viral RNA counts sediments ahead of the peak and a small fraction even co-sediments with 32S ribosomal precursor RNA.

To further probe the size of these RNA species, polyacrylamide gel electrophoresis was undertaken. Fig. 8 shows the electrophorograms of RNA labeled for 30 min and 300 min from 40 hours after infection. Fig. 9 shows the electrophorograms for nuclear and cytoplasmic RNA labeled for 5% min from 40 hours after infection. When cells are labeled for 30 min, 95% of the viral RNA is in the nuclei, judging from a separate experiment. As expected, the patterns of viral RNA in Fig. 7a and 6a are very similar. In Fig. 8a, about 85% of the total viral RNA migrated in two groups between 18S and 28S ribosomal RNA markers. The remainder of the viral RNA migrated more slowly than 28S ribosomal RNA. The mean molecular weights of the two groups are $6.4 \times 10^6$ (Fr. 34) and $5.4 \times 10^6$ (Fr. 46). The profiles are reproducible, suggesting that they are produced by mixtures of characteristic viral RNA components.

When RNA was labeled for 300 min (Fig. 8b) or when RNA was extracted from
The sedimentation profiles of RNA prepared from uninfected and SV 40-infected BSC-1 cells are shown in fig. 7.

In fig. 7, RNA was labeled with 3H-uridine from 10 to 11 hours after infection and 5 x 10^-5 M 5-fluorodeoxyuridine (FUDR) was present in the medium from 9 to 11 hours after infection to prevent viral DNA replication; this is "early" RNA. The counts hybridizable with SV 40 DNA were 0.02% of the total counts. The main peak of RNA hybridizable with SV 40 DNA sedimented together with 18S ribosomal RNA marker and therefore its molecular weight is approximately 6.5 x 10^6 daltons. There is also a shoulder on the faster side of this main peak and a minor peak on the slower side. The approximate molecular weight of the latter is 2.3 x 10^6 daltons. Approximately one-third of the total hybridizable counts sediment very fast. The estimated molecular weight of this fraction of RNA ranges between 2 x 10^6 and 6 x 10^6 daltons, corresponding in size to more than one genome length. The sedimentation profile of hybridizable RNA from cells unexposed to FUDR and labeled from 10 to 11 hours after infection was essentially the same as fig. 7b (data not shown).

When SV 40-infected cells are labeled with 3H-uridine (fig. 7c) from 49 to 50 hours after infection, 5% of the 3H counts in the extracted RNA hybridized with SV 40 DNA; this is the "late" RNA. The position of the peak of viral RNA again corresponds approximately to 6.5 x 10^6 daltons. A significant fraction of the viral RNA counts sediments ahead of the peak and a small fraction even co-sediments with 28S ribosomal precursor RNA.

To further probe the size of these RNA species, polyacrylamide gel electrophoresis was undertaken. Fig. 8 shows the electrophoreograms of RNA labeled for 30 min and 300 min from 40 hours after infection. Fig. 9 shows the electrophoreograms for nuclear and cytoplasmic RNA labeled for 75 min from 40 hours after infection. When cells are labeled for 30 min, 90% of the viral RNA is in the nucleus. The remainder of the viral RNA migrated more slowly than 28S ribosomal RNA. The mean molecular weights of the two groups are 8.4 x 10^6 (Fr. 34) and 5.4 x 10^6 (Fr. 48). The profiles are reproducible, suggesting that they are produced by mixtures of characteristic viral RNA components.

When RNA was labeled for 300 min (fig. 8a) or when RNA was extracted from
the cytoplasm labeled for 75 min (fig. 8a), the fraction of radioactivity in components larger than $8.4 \times 10^5$ daltons M.W. is reduced and the fraction of radioactivity in the smaller components increases. In addition, in fig. 8b a considerable fraction of radioactivity migrated faster than 18S ribosomal RNA.
4. DISCUSSION

The existence of the viral RNA found in SV3T3 cells which is larger than a single strand of SV40 DNA may be explained a priori by either of the following two models. According to the first model, the viral RNA contains all base sequences corresponding to the whole single strand of SV40 DNA. In this model, the RNA base sequences from the "late" genes must not only be prevented from functioning as messenger RNA, but must also quickly be degraded to account for the results of the previous hybridization competition experiments (Oda and Dulbecco, 1968; Aloni et al., 1969). According to the second model, the large viral RNA contains base sequences corresponding only to the "early" genes. To account for the fast sedimentation in DMSO and the slow migration in polyacrylamide gel the RNA must either contain tandem copies of the "early" gene sequence or a composite molecule in which a single copy of the "early" gene sequence is linked to some other molecular species, perhaps cellular RNA. Formation of such a "hybrid" RNA molecule in SV3T3 cells may be justified by the work of Sambrook et al. in which they showed that the viral genome is linked to cellular DNA by bonds resistant to both detergent and alkali (Sambrook et al., 1968).

The experiments reported here disfavor the first model in which all SV40 base
sequences are represented in the large RNA. These experiments also extend the conclusion drawn by Oda and Dublecco and Aloni et al. (Oda and Dublecco, 1968; Aloni et al., 1968) on the stable viral RNA to the large unstable viral RNA described in SV 3T3 cells. The present results, together with the finding of Lindberg and Darnell (Lindberg and Darnell, 1970) present a clear example of post-transcriptional processing of miRNA analogous to that of ribosomal RNA (see, for example, Penman et al., 1989). The similar processing of miRNA does not seem to exist in case of bacteriophage T7 and φ X174 (Smillie, 1975; Hayaishi, personal communication). In contrast, it may be common to many eukaryotic messenger RNA (Gueorguie and Darnell, 1970; also see below). If this proves to be a general phenomenon, it could provide a regulatory role over the differential expression of cellular genes in eucaryotes.

The results reported here do not discriminate between the two alternatives presented in the second model, that is, between tandem "early" genes or composite molecules of viral and cellular material. In a preliminary experiment, enriched "large" and "small" viral RNA were partially degraded and the hybridization properties of the degradation products were examined. The result suggests that the "large" viral RNA contains RNA species of nonviral origin.

The finding of viral RNA in the "early" lytic infection which is considerably larger than one-half of a single strand of SV 40 DNA (2.3 × 10^9 daltons or larger shown in fig. 7b) was unexpected in the light of previous competition experiments (Oda and Dublecco, 1968; Aloni et al., 1968). The two models presented above to explain the "large" RNA in SV 3T3 cells also applies to the large viral RNA of early lytic infection. However, tandem "early" sequences seem to be unlikely because it would require tandemly "early" genes in the template DNA. Formation of such a viral DNA structure at the "early" phase of lytic cycle appears unlikely.

In the "late" cycle, the ratio of the large (larger than 8.4 × 10^9 daltons) to the small RNA species (smaller than 6.0 × 10^8 daltons) is less when whole cells are labeled for a longer time or when RNA was extracted from the cytoplasm as opposed to the nucleus, suggesting another case of post-transcriptional processing. Further work is necessary in order to understand the exact relationship among the characteristic viral RNA species.

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