Sequential Transcription-Translation of Simian Virus 40 by Using Mammalian Cell Extracts

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Ribonucleic acids (RNAs) transcribed in vitro by using the whole-cell extract system of Manley et al. (Proc. Natl. Acad. Sci. U.S.A. 77:3855–3859, 1980) were tested for their efficiency and fidelity in directing protein synthesis in reticulocyte lysates. Simian virus 40 deoxyribonucleic acid (DNA), cleaved by various restriction endonucleases, was used as the template. Successful translation of the small tumor antigen t, as well as the capsid proteins VP1, VP2, and VP3, was detected by immunoprecipitation analysis. Although no synthesis of large T antigen was detected, use of this technology allows detection of large T synthesis resulting from the correct splicing of as little as 0.2% of the in vitro RNA transcripts, making it ideal for use as an in vitro splicing assay. Transcripts synthesized in vitro were used as messages at least as efficiently as were viral messenger RNA's (mRNA's) synthesized in vivo; and in the case of small t, there was more efficient translation of small t mRNA synthesized in vitro than of small t mRNA synthesized in vivo. The transcripts that served as mRNA's for the various polypeptides were identified by using the following two criteria. (i) The sensitivity of synthesis of a given protein to digestion of the template DNA with restriction enzymes allowed the localization of the promoter and coding regions. (ii) Translation of size-fractionated RNA allowed confirmation of the transcript-mRNA assignments. With these techniques, we found that VP2, VP3, and, in some cases, VP1 synthesis resulted from the initiation of translation at internal AUG codons. In fact, families of polypeptides were produced by initiation of translation at AUG codons within sequences coding for VP1 and T, presumably as a result of transcription initiation events that generated 5' ends immediately upstream from these AUGs. Application of this technology for the identification of coding regions within cloned DNA fragments is discussed.

Coupled in vitro transcription and translation systems have aided considerably in elucidating the molecular biology of procaryotes (32). There has been limited development of coupled transcription and translation systems for mammalian cells, however, owing to the lack of an in vitro reaction that initiates transcription on exogenous deoxyribonucleic acid (DNA). Several transcription extracts have recently been developed. The first is that of Weil et al. (31), in which purified ribonucleic acid (RNA) polymerase II is mixed with a cytoplasmic extract; the second is that of Manley et al. (18), in which a whole-cell extract (WCE) of HeLa cells, containing endogenous polymerase II, is used. In both extracts, transcription initiates at specific promoter sites on exogenous templates, and 5' termini of transcripts are capped.

Mammalian in vitro translation systems have been highly developed. The most useful and active of these is the reticulocyte system of Pelham and Jackson (23). Micrococcal nuclease digestion of rabbit reticulocyte extracts renders such extracts exquisitely sensitive to small amounts of exogenous messenger RNA (mRNA) from virtually any source (17). The low premature termination frequency and ease of manipulation are additional appealing characteristics. Sequential transcription by the HeLa WCE and translation by the reticulocyte extract offered promise for development of an in vitro mammalian system for the synthesis of polypeptides from exogenously added DNA. We have successfully explored this possibility, using simian virus 40 (SV40) DNA as the exogenous template.

SV40 is known to express five polypeptides during lytic infection. Two of these are from the early region: large and small tumor antigens, T and t, respectively; three are from the late region

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and are virion components: VP1, VP2, and VP3 (for a review, see reference 16). All of these proteins except T are encoded in colinear viral DNA sequences. Synthesis of a functional mRNA for the 94K T antigen requires RNA splicing to remove intervening sequences containing termination codons. The structure of mRNA's for both early and late SV40 mRNA's has been extensively studied (for a review, see reference 16). Both early polypeptides are translated from mRNA's that sediment at 19S and have common 5' and 3' sequences but differ in terms of sequences excised by splicing. VP2 and VP3 late proteins are encoded in 19S mRNA's as well, whereas the virion protein VP1 is translated from 16S mRNA's (25, 26). The late 19S and 16S mRNA's have coterminal polyadenylated 3' termini and have a heterogeneous set of spliced leader segments. The heterogeneity of leader segments has made it impossible to definitively pinpoint a particular mRNA sequence as being responsible for synthesis of a late polypeptide.

The WCE transcribes both early and late regions of SV40 (9). Major transcripts from the early region are initiated between 0.67 and 0.655 map units (m.u.) at about 10 different sites, most of which correspond to sites of initiation of transcription during infection. Whereas early mRNA's from early infected cells have a majority of their 5' termini at two clustered positions, some three to eight nucleotides downstream from the *BglI* site at 0.67 m.u., the WCE generates a more widely distributed set of 5' termini (U. Hansen, D. G. Tenen, D. M. Livingston, and P. A. Sharp, manuscript in preparation). There are at least two major initiation sites for the WCE transcription in the late region of SV40 as well. These map at positions of 0.685 and 0.72 m.u., and again correspond to positions of 5' termini of mRNA from infected cells. None of the promoter sites on SV40 is particularly efficiently utilized in the WCE system. The early and late regions generate equivalent amounts of RNA, each class being about fivefold less active than the late promoter of adenovirus. RNAs transcribed from both the early and late regions of SV40 have been examined by hybridization techniques for modification by RNA splicing or by cleavage and polyadenylation. As yet, neither post-transcriptional reaction has been observed (9).

Translation of RNA transcribed in vitro from papovavirus DNA has previously been investigated by several groups. Crawford and Gesteland (5) found that the major late polyoma virion protein VP1 could be translated from RNA transcribed by *Escherichia coli* RNA polymerase. More recently, Roberts et al. (28) and

Rozenblatt et al. (29) developed several coupled cell-free systems which expressed SV40 virion proteins. These systems used either *E. coli* RNA polymerase or wheat germ RNA polymerase II to transcribe exogenous DNA and were coupled to wheat germ extracts for translation. Neither RNA polymerase has a simple pattern of specificity for initiation of transcription. Nevertheless, all three SV40 virion polypeptides were identified in wheat germ translation reactions of RNA transcribed from SV40 DNA by wheat germ polymerase II.

MATERIALS AND METHODS

Cells and virus. SV40, strain 777, was grown on the CV1 strain of monkey kidney cells in Dulbecco modified Eagle medium with 10% calf serum. Virus stocks were prepared by passage on CV1 cells at 10^{-3} to 10^{-4} plaque-forming units per cell and titered as described by Uchida et al. (30). All infections for preparation of RNA or DNA were carried out at a multiplicity of infection of 1 to 10 plaque-forming units per cell in Dulbecco modified Eagle medium plus 2% calf serum. ^{35}S-labeled SV40 virions were prepared in vivo by the procedure for adenovirus (24). Mature virions were separated from incomplete or empty capsids and used as markers (22).

DNA and RNA preparations. DNA was prepared from infected CV1 cells harvested at 48 h postinfection by the method of Hirt (10). The form I DNA was purified through two ethidium bromide-cesium chloride density gradients, extracted with isopropanol, and dialyzed against 10 mM tris(hydroxymethyl)-aminomethane (pH 8.5)–1 mM ethylenediaminetetraacetic acid (EDTA). In vivo-synthesized total cytoplasmic RNA was prepared from infected cells harvested at 48 h postinfection by the method of Berk and Sharp (1).

In vitro transcriptions. Standard reaction mixtures contained 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid), pH 7.9; 40 mM KCl; 4.7 mM $MgCl_2$; 0.6 mM EDTA; 0.9 mM dithiothreitol; 6.7% glycerol; 100 μM adenosine triphosphate, guanosine triphosphate, and cytidine triphosphate; 5 μM uridine triphosphate; 10 mM creatine phosphate; 35 μg of SV40 DNA per ml; and 20 μl of HeLa WCE, in a final volume of 50 μl. Ocasionally the 35 μg of SV40 DNA per ml was replaced by 5.0 μg of SV40 DNA per ml plus 14 μg of polydeoxyinosinic-polydeoxycytidylic acid·polydeoxyinosinic-polydeoxycytidylic acid per ml (kindly supplied by W. McClure). Reactions were incubated for 60 min at 30°C and then extracted as described by Manley et al. (18).

To directly monitor in vitro RNA products, 10 μCi of uridine [α-^{32}P]triphosphate was included in a 25-μl reaction. The extracted RNA was glyoxylated as described by McMaster and Carmichael (20) and electrophoresed through a 1.4% agarose gel in 10 mM sodium phosphate (pH 6.8)–3 mM EDTA–0.1% sodium dodecyl sulfate for 3.5 h at 6 to 7 V/cm.

Quantitation of RNA. Two DNA probes were used for hybridizations to RNA: (i) SV40 DNA was cleaved with *BclI*, labeled at the 5' end with polynucleotide kinase and adenosine [γ-^{32}P]triphosphate (19),

and then cleaved with BamHI. The mixture of two fragments was used as the long probe. (ii) SV40 DNA was cleaved with AvaII, labeled at the 5' end with polynucleotide kinase and adenosine [γ-^{32}P]triphosphate (19), and fractionated on a 5% acrylamide gel. The AvaII D fragment was cut out, electroeluted from the gel, and digested with PvuII. The digest was denatured and separated on a 5% acrylamide strand separation gel (19). The labeled 380-base single strand was used as the short probe.

The long probe at 1.5 µg/ml was hybridized with RNA in a 15-µl reaction of 80% formamide–0.4 M NaCl–40 mM piperazine-N,N'-bis(ethanesulfonic acid)(pH 6.5)–1 mM EDTA, as described by Berk and Sharp (2), with a 5-h incubation at 49°C. S1 nuclease digestions were performed as described (2), using 500 U of S1 nuclease (Miles Laboratories, Inc.) and digesting at 45°C for 30 min. The short single-stranded probe at 60 ng/ml was hybridized with RNA in a 15-µl reaction containing 0.75 M NaCl, 50 mM HEPES (pH 7.0), and 1 mM EDTA for 5 h at 50°C. S1 nuclease digestions were performed with 2,000 U of S1 nuclease, digesting at 30°C for 1 h. The reactions were, in all cases, extracted with phenol and then with chloroform. The samples with which the long probe was used were separated by electrophoresis through a neutral 1.4% agarose gel (2); the samples with which the short probe was used were separated by electrophoresis through an 8.3 M urea–8% acrylamide gel (0.3 mm thick) (19).

In vitro translations. The reticulocyte lysate in vitro translation system of Pelham and Jackson (23) was used for all translations. The potassium acetate concentration added to the translation was 80 mM, and the magnesium acetate concentration was 0.65 mM. The translations were carried out for 60 min at 30°C. Amersham translation-grade [^{35}S]methionine (800 to 1,300 Ci/mmol) was used in the translations at concentrations of 25 to 40 µCi per 25-µl reaction. For in vitro translations that served as positive controls, 20 µg of in vivo-synthesized total cytoplasmic RNA from SV40-infected CV1 cells was used for each 25-µl translation reaction. When in vitro-transcribed material served as the source of RNA, translation was programmed with RNAs resulting from one 50-µl transcription reaction. Before translation, ethanol-precipitated in vivo- or in vitro-transcribed RNA was pelleted by centrifugation for 10 min at 15,000 rpm in an Eppendorf centrifuge and subsequently washed twice by blending in a Vortex mixer and recentrifuging the pellet in 0.4 M sodium acetate in 66% ethanol. After one final wash of the pellet in 66% ethanol, each pellet was air-dried and suspended in 5 µl of deionized water for one 25-µl reticulocyte translation reaction.

Methyl mercury gel electrophoresis. Methyl mercury gel electrophoresis was used to separate the in vitro-transcribed RNAs based on size (21). A 1.5% low-melt agarose gel was run for 14 to 19 h at 2.5 V/cm. Eight 50-µl transcription reactions were fractionated into 45 to 50 3-mm-wide fractions after neutralization of the methyl mercury. The RNA was extracted from the gel slices in the presence of 40 µg of carrier yeast transfer RNA by heating the gel fractions in 0.5 ml of 0.5 M ammonium acetate with 2 mM EDTA in a microwave oven. Subsequent phenol and chloroform extractions were carried out as described previously (21). One-third of the RNA in each slice was used for

one 25-µl translation and the subsequent immunoprecipitation.

Antisera, immunoprecipitations, and gel electrophoresis. Several batches of antitumor sera were tested, and all gave essentially identical results. The antisera used for all of the T antigen experiments shown here were generously donated by David Livingston and George Diamandopoulos. The anti-VP3 serum was the generous gift of Harumi Kasamatsu. The anti-sodium dodecyl sulfate-denatured SV40 virion serum was kindly donated by Carol Prives and is described in Prives and Shure (26). The immunoprecipitations were carried out by the Staphylococcus aureus method of Kessler (12). The translation reactions were diluted fivefold in RIPA buffer [150 mM NaCl, 20 mM tris(hydroxymethyl)aminomethane, pH 7.4, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton X-100, 5 mM EDTA, and 0.05% sodium azide], and this and all subsequent steps were carried out at 4°C. A preclear incubation was carried out for 16 to 20 h at 4°C by adding the protease inhibitor phenylmethylsulfonyl fluoride to 1 mM, nonimmune horse, mouse, hamster, or rabbit serum, and 100 µl of a 10% S. aureus suspension to each 25-µl reaction. After this incubation, the reactions were centrifuged at 15,000 rpm for 15 min in an Eppendorf microfuge. The precleared supernatant was then incubated with specific serum for 2 to 8 h before addition of 10 times the serum volume of a 10% suspension of S. aureus and a further incubation for 1 h. The S. aureus pellets were then washed twice with RIPA buffer containing 0.5 M NaCl and once with RIPA buffer containing 0.15 M NaCl. The final washed pellets were extracted with 50 µl of sample buffer for electrophoresis and boiled for 5 min. The supernatants were electrophoresed on standard Laemmli sodium dodecyl sulfate gels (15) containing 15% acrylamide and 0.086% N,N'-methylene bisacrylamide. The gels were then processed by using the fluor Enhance from New England Nuclear Corp. and dried. The dried gels were exposed on Kodak XR-2 film that was preflashed as described by Bonner and Laskey (4).

Quantitation. Quantitation of bands on autoradiograms was accomplished by scanning with a Zeineh laser densitometer.

RESULTS

In vitro transcription of specific SV40 RNAs. Handa et al. (9) have shown that SV40 DNA is an efficient template for in vitro transcription by WCEs prepared from HeLa cells by the method of Manley et al. (18). The predominant RNA products from transcription of SV40 DNA by WCE are initiated in the 0.67- to 0.65-m.u. region from the E strand and in the 0.685 to 0.72-m.u. region from the L strand. The 5' termini of these major transcripts have been mapped to within a few nucleotides (Hansen et al., in preparation). In general, the WCE system yields about equal amounts of RNA from the E and L strands. The RNA products are neither processed by post-transcriptional cleavage and polyadenylation nor by splicing, although, in the case in which it has been examined, the RNA

products are capped. Examples of transcripts generated from the DNA templates used in this study are shown in Fig. 1. Incorporation of α-^{32}P-labeled uridine triphosphate was used to monitor the transcription as shown in this figure; the input RNA for all translation experiments, however, was unlabeled. The runoff transcripts from EcoRI-cleaved SV40 DNA were resolved in the gel shown in Fig. 1. RNAs of approximately 3,450 nucleotides and 3,400 nucleotides long were transcribed after initiation on the E strand at positions 0.67 and 0.655 m.u., and RNA products of lengths of 1,640 nucleotides and 1,490 nucleotides were transcribed after initiation at 0.685 and 0.72 m.u. on the L strand. Runoff products from other restriction endonuclease-cleaved SV40 templates can be resolved as shown in Fig. 1, confirming the identity of these in vitro products. Less abundant RNA products can also be seen throughout the gel, indicating that WCE transcription of SV40 was not completely specific. These minor products resulted from RNA polymerase II transcription, as their synthesis has been shown to be sensitive to low concentrations of α-amanitin.

Translation of proteins specified by the early region was tested by using RNA transcribed from SV40 DNA cleaved by either EcoRI, PstI, or TaqI at 0.0, 0.27, or 0.57 m.u., respectively. RNA transcribed in a 50-μl WCE mixture was extracted with organic solvents, precipitated with ethanol, and used to program a reticulocyte translation system. The total translation products directed by the RNA in the transcription reaction, as well as by cytoplasmic RNA from SV40-infected CV1 cells, can be seen in Fig. 2, lanes A through F. A broad spectrum of products is visible in these lanes owing to the presence of HeLa cell mRNA's in the WCE. No proteins dependent on the presence of SV40 DNA in the transcription reaction can be seen above the HeLa cell background (cf. lane A with lane B through E). However, when a longer gel was used to fractionate the products, as is shown in Fig. 3, a strong band the size of small t could be seen in the products derived from the RNAs transcribed in vitro (except when TaqI-cleaved DNA was used as a template).

In vitro translation of polypeptides related to early proteins of SV40 was detected by immunoprecipitation with antitumor serum. Lanes B', C', and D' of Fig. 2 show the immunoprecipitated products from RNA transcribed from EcoRI-, TaqI-, and PstI-cleaved SV40 DNA, respectively. Cytoplasmic RNA from SV40-infected cells stimulated synthesis of T (94,000 molecular weight [94K]) and t (16K) as expected (lane E'). A prominent band comigrating with t (16K) was also translated from RNA transcribed from the
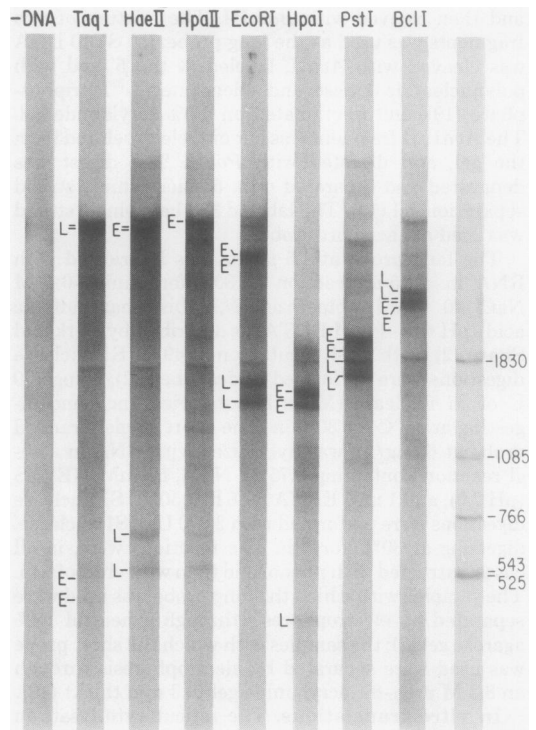

FIG. 1. In vitro transcription of SV40 DNA cleaved with various restriction endonucleases. SV40 DNA was cleaved by the indicated restriction endonucleases and transcribed in the HeLa WCE system of Manley et al. (18), with some slight modifications as described in the text. Uridine [α-^{32}P]triphosphate was used to label the RNA. Approximately one-fifth of each RNA sample was glyoxylated and fractionated on a 1.4% agarose gel in 10 mM sodium phosphate buffer, pH 6.8. The gel was dried and exposed for 1 day on a preflashed Kodak XR-5 film. E indicates the early SV40 RNA, and L indicates the late SV40 RNA. The markers are fragments of HinfI-cleaved SV40 DNA, which were labeled at the 5' end and glyoxylated. The sites of cleavage on the SV40 genome by the restriction endonucleases are as follows: TaqI, 0.56; HaeII, 0.82; HpaII, 0.73; EcoRI, 1.0; HpaI, 0.17, 0.37, and 0.76; PstI, 0.27 and 0.04; and BclI, 0.19. The length of the expected runoff products can be calculated by recalling that the early promoters map at 0.67 and 0.665 and the late promoters map at 0.72 and 0.685. SV40 is 5,243 nucleotides long. The sites can be seen in the context of the early and late promoters in Fig. 2 and 3.

EcoRI- (lane B') and PstI-cleaved (lane D') templates. However, a protein of 16K was not translated from RNA of TaqI-cleaved DNA, but a less prominent band of 14K was obtained. This was expected, as TaqI cleaves SV40 DNA about 100 nucleotides upstream from sequences coding for the carboxyl terminus of t (Fig. 2). The dramatic reduction in the amount of t-related
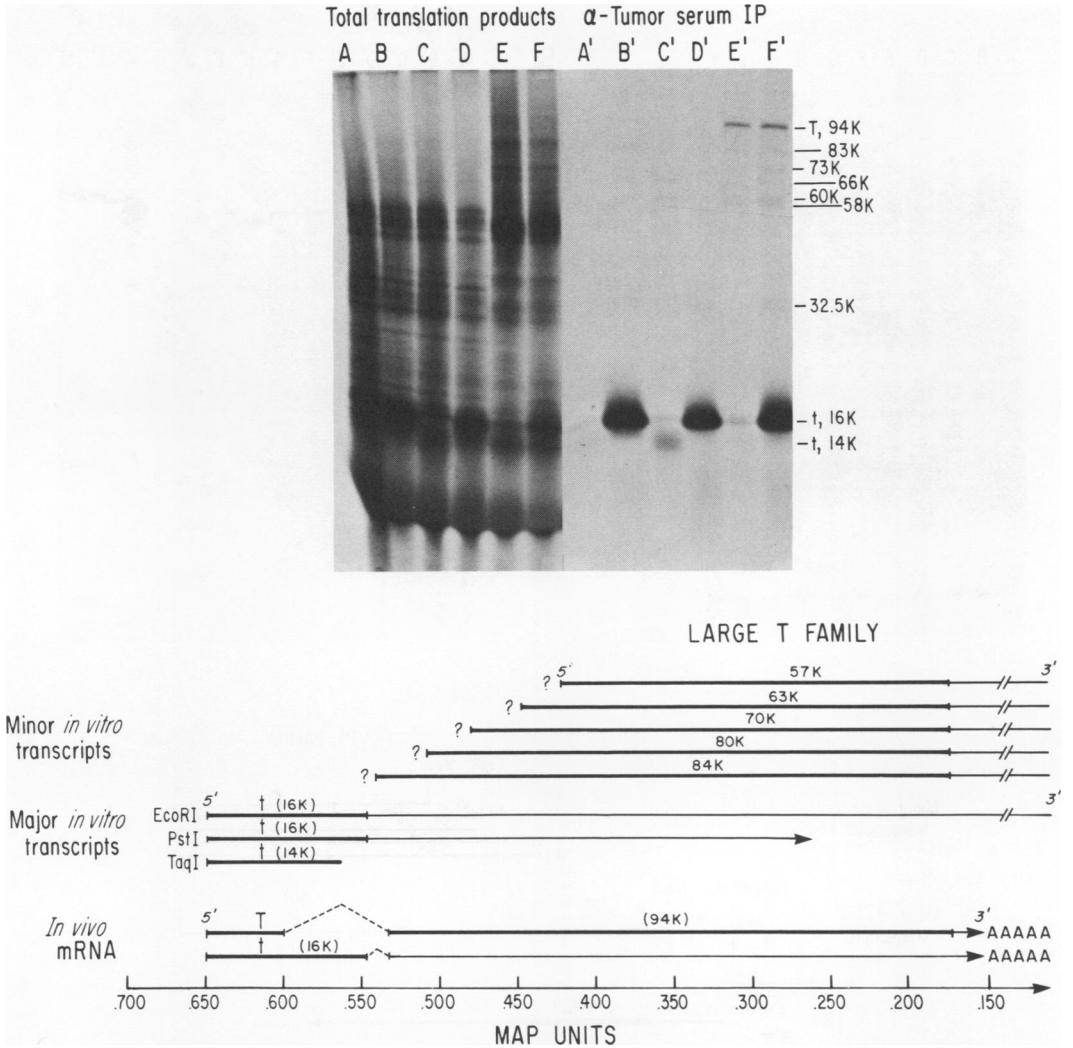
FIG. 2. *Translation of early SV40 proteins from RNA transcribed in vitro. In vitro transcription of SV40 DNA by the HeLa WCE system of Manley et al. (18) was carried out in 50-μl transcription reactions programmed by 1.75 μg of SV40 DNA. The transcription reaction was extracted with phenol, precipitated with ethanol, and subsequently translated in one 25-μl reticulocyte translation reaction. The non-immunoprecipitated translation products (1/15 of the entire translation reaction) were fractionated on a 15% acrylamide gel, as shown in lanes A through F. The results of immunoprecipitation of 20 μl of each translation reaction by anti-SV40 tumor serum are shown in lanes A' through F' (corresponding to lanes A through F). The sources of RNA for the translations were as follows: (A) RNA from an in vitro transcription reaction containing no exogenous DNA; (B through D) RNA from in vitro transcription of SV40 DNA cleaved with EcoRI (B), TaqI (C), or PstI (D); (E) 20 μg of total cytoplasmic RNA synthesized in vivo by SV40-infected CV1 cells; (F) A 1:1 mixture of the RNAs described for lanes B and E. The fluorographed gel was exposed for 3 days on preflashed Kodak XR-5 film. The data depicted in the diagram are taken from Lebowitz and Weissmann (16). Heavy lines indicate the coding regions for the various proteins. The molecular weights listed were those observed based on standards of bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K), lactoglobulin A (18K), and cytochrome C (12K).*

polypeptide from *Taq*I-cleaved DNA as compared with either *Eco*RI- or *Pst*I-cleaved DNA is probably a result of the absence of a nonsense codon at the end of the transcript (see below).

Lack of synthesis of large T. A polypeptide comigrating with the 94K T of SV40 was not present in products transcribed from either *Eco*RI-, *Pst*I-, or *Taq*I-cleaved DNA. This would not be expected for RNA transcribed from either *Pst*I- or *Taq*I-cut DNA, as these endo-
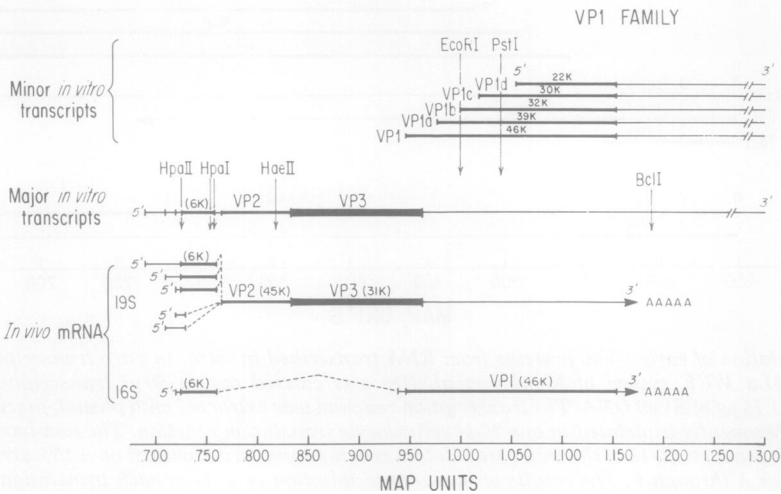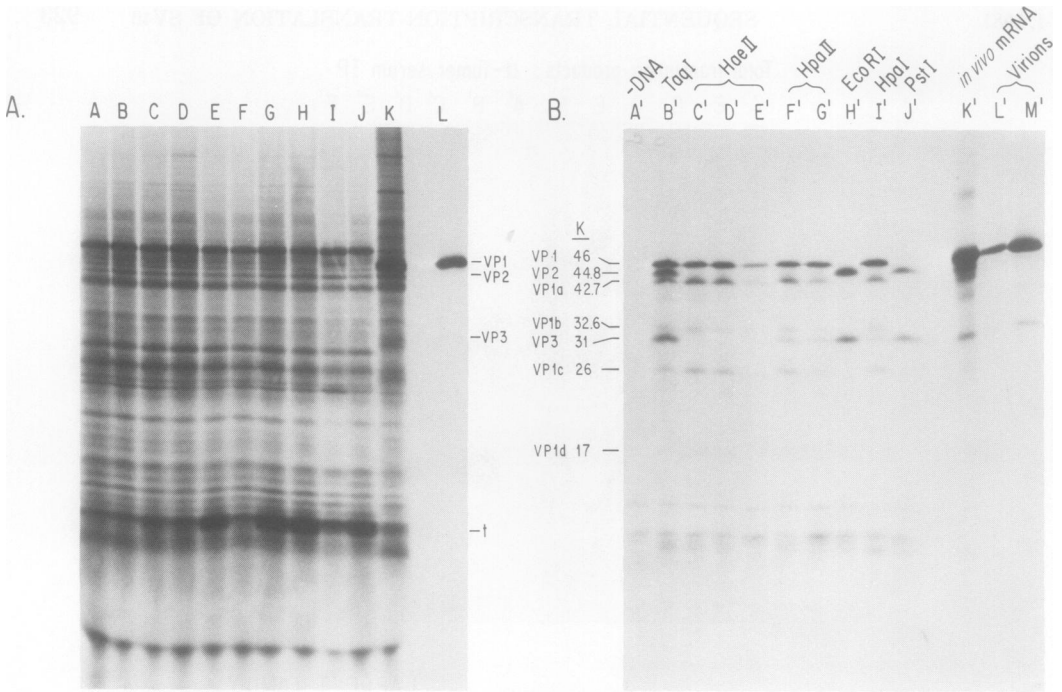
FIG. 3. *Translation of SV40 late proteins from RNA transcribed in vitro. (A) In vitro transcriptions and translations were carried out as described in the legend to Fig. 2. Lanes A through K depict the total translation products programmed by RNA from: (lane A) an in vitro transcription reaction containing no exogenous DNA; (lanes B through J) in vitro transcription reactions containing SV40 DNA cleaved by TaqI (B), HaeII (C through E), HpaII (F and G), EcoRI (H), HpaI (I), or PstI (J); (K) 20 µg of total cytoplasmic RNA harvested from SV40-infected cells 48 h postinfection. The transcription reactions that resulted in the proteins shown in lanes E and G were carried out in the presence of alternating polydeoxyinosinic-polydeox-ycytidylic acid. (B) The results of immunoprecipitation of the translation products by anti-sodium dodecyl sulfate denatured SV40 virion serum in combination with anti-VP3 serum are shown in the corresponding lanes A' through K'. Purified SV40 virions are shown in lane M' and the immunoprecipitated products of the purified virions are shown in lane L'. The data shown in the diagram were taken from a review by Lebowitz and Weissmann (16) and the references concerning SV40 late mRNA contained therein. Only the major spliced forms of the 19S and 16S classes of mRNA are shown; other minor species have been described. Heavy lines indicate the coding region for the various late proteins. Molecular weights were assigned based on the migration of SV40 virion proteins as well as the standard marker proteins shown in Fig. 2.*

nucleases cleave within the large T-coding region of SV40. However, if a small fraction of the RNA transcribed from the EcoRI-cleaved DNA had been processed by excision of the intervening sequences between 0.60 and 0.53 m.u., synthesis of T (94K) would have been possible. An estimate of the maximum level of such spliced RNA that could have been detected among sequences transcribed from EcoRI-cleaved DNA was determined by densitometer tracing of the gel shown in Fig. 2. A correction for the number of [^{35}S]methionines in t and T polypeptides was made; the resulting calculation suggests that fewer than 1 in 500 of the RNAs transcribed from EcoRI-cleaved DNA was processed in a fashion permitting synthesis of the 94K T antigen. As a control to prove that T polypeptide synthesis was possible in lysates containing RNA from WCE, a 1:1 mixture of RNA from SV40-infected cells (which alone gave rise to the products shown in lane E') and RNA from EcoRI-cleaved DNA (lane B') was translated and immunoprecipitated. The products of this translation are shown in lane F', in which it can be seen that WCE RNA in no way inhibited the synthesis of large T.

Synthesis of the large T family of polypeptides. A series of bands that migrated as polypeptides of 60K to 80K were immunoprecipitated from RNA transcribed from EcoRI-cleaved DNA (lane B'). These bands were not present among the translation products of control RNA extracted from the WCE (lane A'), although two of the bands were present among the products of in vivo mRNA. Polypeptides with these molecular weights could be synthesized by initiation of translation at AUG codons downstream from position 0.54 m.u. on SV40, followed by elongation to the termination codon at position 0.17 m.u. (Fig. 2). Such AUG codons are found at 0.519, 0.480, 0.448, and 0.442 m.u. (6, 27), and initiation of translation at these positions would result in 80K, 71K, 63K, and 57K polypeptides, respectively. There are 13 other AUG codons in the T reading frame from 0.44 to 0.17 m.u. (6, 27). Polypeptides corresponding to initiation at these codons were not observed. To confirm the proposed identity of the 60 to 80K bands as large T-related polypeptides, translation and immunoprecipitation were performed by using RNA from a PstI-cleaved template. PstI cleaves SV40 DNA at 0.27 m.u., in the middle of the coding region for these products. RNA transcribed from this template did not program the synthesis of the 60 to 80K series of polypeptides (lane D'). It is also of interest that some members of this series of polypeptides were translated from the RNA products of TaqI-cleaved SV40 DNA, suggesting

that the transcripts responsible for their translation were initiated downstream from the TaqI cleavage site at 0.57 m.u. Thus, at least some of the translation of the 60 to 80K polypeptides probably resulted from initiation of transcription by RNA polymerase II at sites other than the in vivo sites in the vicinity of 0.66 m.u. A similar situation was observed for synthesis of VP1-related polypeptides from the late region of SV40 (see below).

Quantitation of relative efficiencies of in vivo- and in vitro-synthesized RNA. Since the structure of the RNAs synthesized in vitro is quite different from that of the in vivo mRNA's with respect to the 3' terminus, polyadenylation, splicing, and possibly the 5'-end structure, one question that can be raised regards their translation efficiency relative to the in vivo mRNA's. The amount of viral RNA in in vitro versus in vivo preparations was compared by hybridizing unlabeled RNA to a 5' end-labeled DNA probe, treating the hybrids with S1 nuclease, resolving the products by gel electrophoresis, and comparing the intensities of the bands on the resulting autoradiogram. Probe DNA labeled at the BclI site at 0.19 m.u. demonstrated that about 0.03% of the in vivo cytoplasmic RNA encoded either T or t, of which 45% represented t mRNA (data not shown). This indicates the presence of 1 to 2 ng of t mRNA per 10 μg of late-infected cytoplasmic RNA. About 0.02% of the extracted in vitro RNA was early SV40 RNA, there having been about 2 ng of early SV40 RNA transcribed in a 50-μl WCE transcription reaction (data not shown). When equivalent amounts of t mRNA were translated (Fig. 2), much more t polypeptide was programmed by the in vitro RNA as compared with the in vivo RNA. One must conclude that in vitro t mRNA is more efficiently translated than is in vivo t mRNA.

Hybridizations of in vitro RNA with a short single-stranded DNA probe allowed a direct comparison of the amounts of early RNA templated by SV40 cleaved with TaqI and that cleaved with HaeII. Equivalent amounts of early RNA were transcribed from the two templates (data not shown). Thus, the lower amount of translated t polypeptide produced from a shortened t mRNA (Fig. 2, lane C') as compared with the amount of full-length t produced from a long RNA (Fig. 2, lane B' and D') may be due to the lack of a termination codon on the shortened RNA, since the same amount of early RNA is available for translation in both cases.

Synthesis of VP1, VP2, and VP3 from RNA transcribed in vitro. The translatability of the in vitro transcripts from the SV40 late region was investigated by procedures similar to

those used for the early region. *Taq*I-cleaved DNA was used as a template to investigate translation of all three virion polypeptides, as it has an intact late region. Templates cleaved by other restriction endonucleases, primarily those cutting SV40 once, were used to examine translation of individual polypeptides. The cleavage sites are shown in Fig. 3.

The total translation products obtained from these in vitro transcripts and from in vivo mRNA isolated late in infection are shown in Fig. 3A, lanes A through K. There were no obvious bands corresponding to VP1, VP2, or VP3 in these total-translation reactions, except in lane K where late in vivo mRNA was translated. As with the early region products, the late polypeptides were detected by precipitation with antiserum. Antisera to sodium dodecyl sulfate-denatured virion polypeptides as well as αVP3 sera were obtained and tested in combination for their specificity. Immunoprecipitation of purified, sodium dodecyl sulfate SDS-denatured virions as well as in vitro translation products confirmed the αVP1, αVP2, and αVP3 specificity of these sera (lane L') as well as their ability to recognize the nonassembled in vitro-translated proteins (lane K').

Immunoprecipitation of the translation products programmed by transcripts of the *Taq*I-cleaved template is shown in lane B', Fig. 3B. By comparing this lane with lane A', where no SV40 DNA was used in the transcription reaction, and lane L', where purified virions were precipitated, it can be seen that the synthesis of all three virion polypeptides was detected by the in vitro-transcribed RNA. In addition to VP1, VP2, and VP3, there was an assortment of bands ranging from 42K to 17K (lanes B' through G' and lane I'), all of which were also present at low levels when in vivo RNA was used (lane K'). Fragmentation of a small percentage of the in vivo mRNA probably accounts for the presence of these bands.

Synthesis of VP1 and VP1 family of polypeptides. By using DNA templates cleaved within the late region, information concerning the identity of the transcripts that served as VP1, VP2, and VP3 messages was obtained. Proof that the 46K protein was indeed VP1 was provided by the absence of this protein when templates were cut with *Eco*RI or *Pst*I, which cleave within the VP1-coding region (lanes H' and J', Fig. 3). A family of bands of 42K, 32K, 26K, and 17K exhibited the same template sensitivity as did VP1, with the exception of the 17K polypeptide when *Eco*RI was used, as discussed below. These bands probably represent a VP1 family similar to the large T family, with each band being the result of initiation at an

AUG distal to the VP1 AUG and termination at the VP1 nonsense codon. The sequence data (6, 27) provide evidence for this, as there are AUGs at 0.98, 0.00, 0.02, and 0.05 m.u. 39K, 32K, 30K, and 22K polypeptides would be the result of translations from these AUG codons. These predicted molecular weights agree quite well with those observed for the three largest polypeptides, which are referred to as the VP1a, VP1b, and VP1c polypeptides, respectively, in Fig. 3B. The presumptive VP1d polypeptide with a predicted molecular weight of 22K migrated as a protein of $\simeq$17K in this gel system. (This band is faint in the print shown in Fig. 3B but is readily visible on longer exposures.) The differential sensitivity of this 17K protein to an *Eco*RI-cut template (lane H', where it was present) versus that of a *Pst*I-cut template (lane J', where it was absent) provides very strong evidence that this band indeed represents the VP1d polypeptide. An *Eco*RI-cut template could provide about 300 nucleotides for initiation of transcription before the VP1d AUG, whereas on a *Pst*I-cut template, only 80 nucleotides would be available. Note that truncated VP1-related polypeptides, i.e., a group of polypeptides lacking the carboxyl terminus, were not synthesized when *Eco*RI- or *Pst*I-cleaved templates were used. This result is quite similar to the results obtained when *Taq*I-cleaved DNA was used as the template for small t or when *Pst*I-cleaved DNA was used for the large T family of polypeptides.

The transcripts from which VP1 and VP1-related polypeptides VP1a through d were translated probably resulted, like those programming the large T family of polypeptides, from infrequent transcription initiation events near an AUG codon. Synthesis of VP1 and VP1a through d was not sensitive to template cleavage by restriction endonucleases that cut between the late promoters at 0.685 and 0.72 m.u. and the sequences coding for VP1 (0.94 to 0.15 m.u.), e.g., *Hae*II (lanes C' through E'), *Hpa*II (lanes F' and G'), or *Hpa*I (lane I') in Fig. 3. Note that synthesis of both VP2 and VP3 was diminished by template cleavage with these restriction endonucleases. In fact, since transcripts from *Hae*II-cleaved DNA stimulated levels of VP1 and VP1a through d comparable to those stimulated by transcripts from SV40 DNA with an intact late region, it is likely that a majority of the transcripts responsible for VP1-related synthesis were initiated shortly upstream from the VP1 initiation codons. This is also suggested by the insensitivity of VP1d translation to template cleavage by *Eco*RI (see above).

Synthesis of VP2 and VP3. The 19S class of late SV40 mRNA's is thought to serve as a message for the synthesis of both VP2 and VP3,

even though translation of the latter protein would require initiation of translation at an internal AUG. Clearly, translation of most mammalian mRNA involves initiation on the first AUG after the capped 5' terminus (for a review and history, see 13 and 14). It is difficult to design a conclusive experiment demonstrating that VP3 is translated by internal initiation on 19S mRNA from infected cells, because this family of mRNA's has a heterogeneous spliced structure, and VP3 is a minor translation product. In addition, the problem of RNA degradation is always difficult to control in in vivo as well as in vitro experiments.

Some idea of the location of the transcriptional initiation sites for RNAs translated into VP2 and VP3 was obtained by translating unfractionated RNA from templates cleaved with different restriction endonucleases. Figure 3, lanes C' through G' and lane I' show that cleavage of SV40 template with either HaeII, HpaI, or HpaII eliminated VP2 translation. Thus, the transcripts for VP2 were initiated upstream of position 0.72 (HpaII) and 0.76 (HpaI), probably at the two prominent late promoter sites. HaeII template cleavage eliminated VP2 synthesis and provided evidence that the 44K protein was indeed VP2, as this enzyme cleaved in the middle of the VP2-coding sequences. In the same lanes it can be seen that VP3 synthesis was reduced threefold upon cleavage by HpaI or HpaII and was completely abolished upon cleavage by HaeII. This absence of transcripts encoding VP3 was expected, as HaeII cleaved in the VP2 coding region immediately proximal to the VP3 initiator AUG. The fact that HpaI or HpaII DNA cleavage reduced VP3 synthesis threefold suggests that a majority of the transcripts responsible for VP3 translation were also initiated in the 0.68 to 0.72 region, upstream from the AUG codon for VP2. The residual transcripts must have been initiated between 0.76 (HpaI) and 0.82 (HaeII).

Size fractionation of RNA synthesized in vitro: direct identification of VP1, VP2, VP3, and t mRNA's. To test further the hypothesis that VP3 can be translated from RNAs initiated at late promoter sites as well as from RNAs initiated downstream from the VP2 initiator AUG, RNA transcribed in vitro was fractionated by size on methyl mercury-containing agarose gels. EcoRI-cleaved DNA was chosen as template to maximize the resolution of RNAs in the predicted size classes. The gel was fractionated into ≃50 3-mm slices: RNAs that differ in size by ≃100 nucleotides can be resolved in this system. The translation products directed by the nonfractionated RNA as well as by the RNA from individual fractions were immunoprecipi-

tated (Fig. 4). The results confirm the above-mentioned hypothesis, as there was no separation of VP2 translation from that of VP3 in the larger size class of RNA; the peak of VP2 and VP3 activity coincided in fraction 13. The fractions that contained larger RNAs, fractions 11 and 12, showed ratios of VP2 to VP3 similar to that observed in the peak fraction. If VP2 was the exclusive result of translation of the largest class of RNAs initiated in the late promoter region, a greater ratio of VP2 to VP3 in these fractions or the total absence of VP3 would have been observed. Also, as predicted by the observed resistance of one-third of the VP3 translation to HpaI and HpaII, VP3 could be translated from a smaller class of RNAs initiated in the 0.76- to 0.82-m.u. region (lanes 15 through 18, Fig. 4).

RNAs transcribed from a BclI-cleaved template were also fractionated on an agarose gel that contained methyl mercury to confirm the above-mentioned observations concerning VP2 and VP3, as well as to identify the transcripts responsible for VP1 and VP1a through d translation. The results of this fractionation (Fig. 5A) were in agreement with the data shown in Fig. 3 and 4 concerning VP2 and VP3. No exclusive translation of VP2 was found in fractions larger than those which gave rise to VP3 synthesis. The size of the transcripts that directed VP2 and VP3 synthesis in fractions 5 through 9 (Fig. 5A) was calculated to be 2,200 to 2,700 nucleotides by comparison with DNA markers run on the same gel. This size was in agreement with the predicted sizes of 2,400 to 2,600 nucleotides for transcripts that originated between 0.68 and 0.72 m.u. and terminated at the BclI cleavage site of 0.19 m.u.

VP1 synthesis was directed by the 2,200 to 2,700-nucleotide class of RNAs as well as by a smaller class of RNAs of 1,300 to 1,700 nucleotides (lanes 13 through 16, Fig. 5A). The VP1 as well as the VP1a polypeptides were predicted to result from translation of the smaller class of RNAs, based on the data shown in Fig. 3 concerning the template sensitivity of these proteins. (The synthesis of VP1 from the larger class of RNAs was not expected and will be discussed further below.) The VP1a through d family of proteins was expected to result from transcripts produced by RNA polymerase II initiation events within the VP1 gene, and should therefore have been translated by smaller transcripts than those encoding VP1. To maximize the resolution of the presumptive VP1 and VP1a RNAs, methyl mercury gel electrophoresis of BclI-directed transcripts was again performed, but for a longer period of time than was used for both the previous gels, with the results shown in
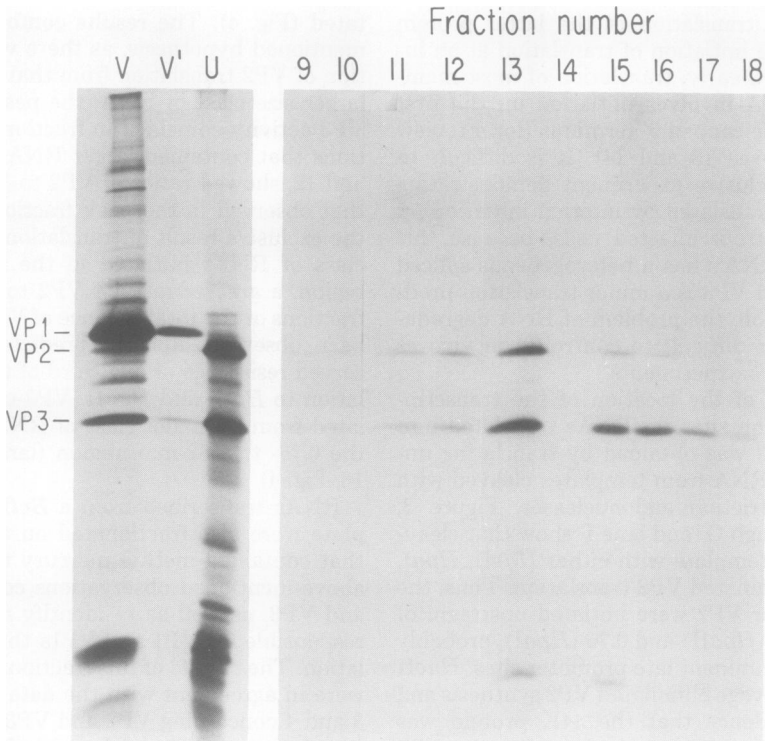
FIG. 4. *Immunoprecipitation of VP2 and VP3 from translation products of size-fractionated transcripts synthesized in vitro from EcoRI-cleaved SV40 DNA. Transcripts synthesized in vitro from an EcoRI-cleaved SV40 template were denatured with methyl mercury and fractionated by size on a 1.5% agarose gel containing methyl mercury. Unfractionated RNA as well as the RNA eluted from the various gel slices was translated, and the resulting products were immunoprecipitated by the anti-SV40 serum described in the legend to Fig. 3. The gel was sliced into 50 3-mm slices, and each slice was assayed by translation and immunoprecipitation. Those fractions containing SV40-specific products are shown. (V) Purified SV40 virion markers; (V') immunoprecipitated SV40 virion markers; (9 through 18) immunoprecipitated proteins resulting from translation of fractions 9 through 18. Fraction 1 was from the top of the gel.*

Fig. 5B. It is clear from these data that the RNAs for VP1 and VP1a are separable RNA species and that VP1a did not result from internal initiation on a VP1 mRNA. The observed size of 1,100 nucleotides for VP1a mRNA matches the expected size of 1,260 to 1,100 nucleotides for an RNA initiated between the VP1 AUG at 0.95 m.u. and the VP1a AUG at 0.98 m.u.

The translation products derived from the transcripts of the *Bcl*I template were also analyzed for the presence of small t by immunoprecipitation with antitumor serum. These results (not shown) indicate that the transcripts of ≃2,600 nucleotides that would result from RNA polymerase II initiation at 0.65 to 0.67 m.u. served as message for small t as predicted by the data shown in Fig. 2.

DISCUSSION

The current technologies of recombinant DNA and in vitro transcription systems provide

powerful tools by which gene expression can be studied. The experiments described here have coupled the existing technology of in vitro translation with these newer techniques to explore further the expression of the much-studied virus SV40. The technology described provides a basis for the application of these techniques to systems in which nucleic acids may be manipulated, as is indeed the case in many systems where the proteins are difficult to study directly.

Efficiency of in vitro RNAs as messages. The translation of RNA molecules synthesized in vitro is apparently a very efficient process in the reticulocyte lysate system. Immunoprecipitation of ^{35}S-labeled polypeptides programmed by in vitro transcripts led to the identification of four of the five known SV40 proteins: VP1, VP2, VP3, and the small tumor antigen t. All of these proteins are encoded in colinear sequences in the viral DNA and do not require RNA splicing to form a translatable RNA. The efficiency of translation in the reticulocyte lysate of in vitro-
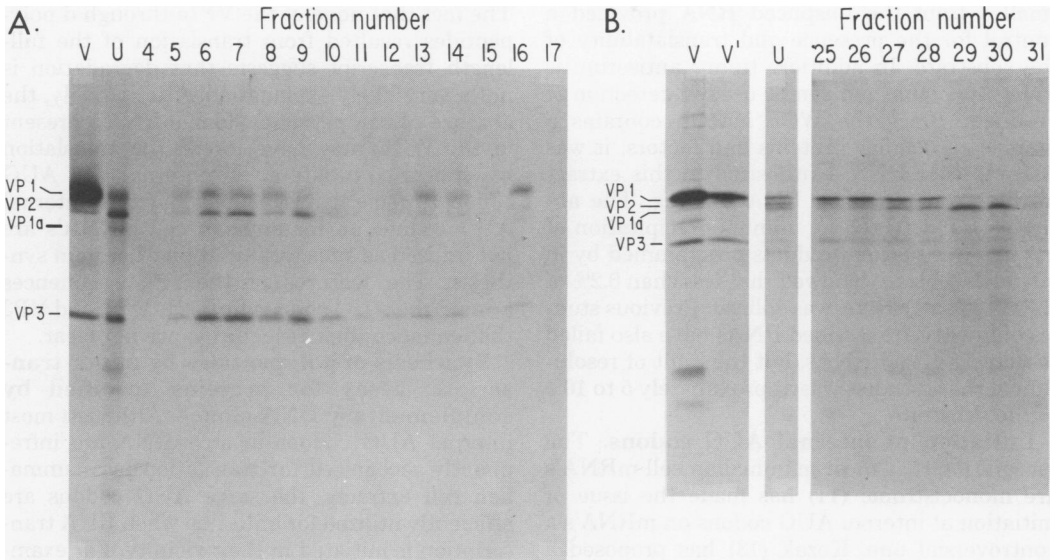
FIG. 5. Immunoprecipitation of VP1, VP1a, VP2, and VP3 from translation products directed by size-fractionated transcripts synthesized in vitro from BclI-cleaved SV40 DNA. RNA synthesized in vitro from BclI-cleaved SV40 DNA was denatured by methyl mercury treatment and subsequently electrophoresed on agarose methyl mercury gels as described in the legend to Fig. 4. (A) Electrophoresis of the methyl mercury-containing agarose gel was carried out at 40 V for 14 h. The gel was sliced into 50 fractions, and the eluted RNA as well as a portion of unfractionated RNA was translated. The translated products were immunoprecipitated with the sera described in the legend to Fig. 3. The immunoprecipitated products are shown from those fractions containing SV40 specific products. (V) Purified SV40 virion markers; (U) immunoprecipitated products of unfractionated RNA; (4 through 17) immunoprecipitated products of fractions 4 through 17. DNA markers indicated that fractions 5 through 9 contained RNAs of 2,200 through 2,700 nucleotides, and fractions 13 through 16 contained RNAs of 1,300 through 1,700 nucleotides. (B) Electrophoresis of transcripts derived from BclI-cleaved DNA was carried out at 40 V for 18 h to maximize fractionation of VP1 and VP1a mRNA's. The protocol described for A (above) and for Fig. 4 was used. DNA markers indicated that RNA of ≃1,100 nucleotides was present in fraction 29.

transcribed RNA was comparable to that of the in vivo mRNA's. As little as 2 ng of RNA from the early region of SV40 stimulated synthesis of the 16K tumor antigen t to a level where it was easily detected over a background of ^{35}S-labeled proteins stimulated by 0.3 µg of polyadenylated HeLa mRNA, i.e., the amount of polyadenylated cellular RNA in a 50-µl WCE reaction mixture. This can be compared with the efficiency of translation of early mRNA extracted from infected cells, in which 30 ng of viral RNA would be required to stimulate the equivalent amount of small t. The high translation efficiency of the in vitro-transcribed early RNA was probably the consequence of some of these RNAs having 5'-termini within 10 nucleotides of the initiation AUG codon (Hansen et al., manuscript in preparation). Presumably, these 5' termini are capped, since the WCE system (18) as well as the system of Weil et al. (31), in which a cytoplasmic extract was used, have been shown to cap 5' termini.

The efficiency of translation of VP2 and VP3 from RNAs transcribed from the late region of SV40 was less than that of the early region. For example, equivalent amounts of RNAs initiated at the late and early promoter sites stimulated about 1/20 the amount of VP2 as compared to small t, respectively. There was nothing peculiar about the in vitro-synthesized RNA from the late region, as in vitro- and in vivo-transcribed late RNAs were about equally efficient substrates for translation. The differences in translation efficiency between early and late RNAs may be due to the location of the 5' ends with respect to the initiation codons. The results shown here were not the result of an exclusive feature of SV40. RNA transcribed in vitro from a fragment of adenovirus 2 DNA has also been observed to be correctly translated to give rise to polypeptide IX (A. Fire and C. Cepko, data not shown).

Assay for RNA splicing. The sensitivity of in vitro translation coupled with the specificity of immunoprecipitation made for an ideal assay for RNA splicing. RNA from the early region of SV40 was an excellent substrate for this assay. The synthesis of the large T polypeptide required excision of the intervening sequences between 0.53 and 0.60 m.u. whereas synthesis of

small t from the unspliced RNA provided a control for the presence and translatability of the substrate. In addition, tumor antiserum is widely available and can be used in detection of products. Since the WCE system contains a number of cellular proteins and factors, it was possible that RNA synthesized in this extract might be modified by RNA splicing. The absence of T detected by immunoprecipitation of in vitro translation products programmed by in vitro RNA clearly showed that less than 0.2% of the SV40 early RNA was spliced. Previous studies of in vitro-transcribed RNAs have also failed to detect spliced RNAs, but the limit of resolution of these studies was approximately 5 to 10% of the substrate.

Initiation at internal AUG codons. The recognition that most mammalian cell mRNA's are monocistronic (11) has made the issue of initiation at internal AUG codons on mRNA's a controversial one. Kozak (13) has proposed a "scanning model" in which initiation factors initially recognize the capped terminus of an mRNA, direct the 30S subunit to move along the RNA in an adenosine triphosphate-dependent fashion, and then begin protein synthesis at the first AUG codon. Translation of both VP2 and VP3 polypeptides from late 19S mRNA suggests that, in some cases, protein synthesis could initiate at internal AUG codons. In fact, translation of VP1, VP2, and VP3 from the most abundant forms of 19S or 16S in vivo mRNA's requires initiation at internal AUG codons. The major late promoter sites for in vitro transcription of SV40 map at 0.72 and 0.685 m.u. (Hansen et al., manuscript in preparation). RNAs initiated at either of these sites have AUG codons before the initiation codons for translation of either VP2 or VP3, respectively (7). Since cleavage of viral DNA with HpaII (0.725 m.u.) eliminated translation of VP2 and reduced translation of VP3 by threefold, it is likely that the reticulocytic system was initiating at internal AUG codons for translation of these two proteins. In addition, fractionation of RNA by electrophoresis in methyl mercury agarose gels also suggested that VP2 and VP3 were translated from the same set of major transcripts, which were intitiated in the 0.685- to 0.72-m.u. region. Some VP1 synthesis was also observed to result from translation of the same transcript as that of VP2 and VP3 when transcripts derived from BclI DNA were fractionated on methyl mercury gels. The synthesis of VP1 from this class of transcripts was not expected. The observation that VP1 could be translated from the large transcript size class after isolation by gel electrophoresis could be the result of RNA degradation or of internal initiation on the fractionated RNA.

The fact that none of the VP1a through d polypeptides resulted from translation of the full-length transcript suggests that degradation is not a very likely explanation. Alternatively, the absence of competing cellular mRNA's present in the WCE may have forced the translation machinery to intiate at the internal VP1 AUG codon. In general, it is obvious that most internal AUG codons on mammalian cell mRNA's are not utilized as sites for initiation of protein synthesis. The features of the RNA sequences around the AUG codons for VP1, VP2, and VP3 that enhance their recognition are not clear.

Synthesis of polypeptides by minor transcripts; assay for proteins specified by complementary DNA clones. Although most internal AUG codons in an mRNA are infrequently recognized for translation by mammalian cell extracts, the same AUG codons are efficiently utilized for initiation when RNA transcription is initiated in their vicinity. For example, infrequent initiation by RNA polymerase II within sequences coding for VP1 produced transcripts that directed the efficient translation of four polypeptides: VP1a, VP1b, VP1c, and VP1d. The same phenomenon was observed for the early region of SV40. The late transcripts responsible for synthesis of VP1a through d were less abundant than those initiated at major late promoter sites by at least a factor of 10, but they were so efficiently translated that their protein products appeared in amounts comparable to VP2. This was probably owing to the close proximity of a 5' terminus to an AUG codon. The level of sensitivity observed here makes it possible in principle to detect proteins encoded by any DNA fragment, including a cloned fragment, if an antibody or an assay for the protein's activity is available.

Lack of termination codon inhibits translation. The data observed in this system indicate that the absence of the proper termination signal, a nonsense codon at the very least, probably prevented an RNA from functioning efficiently as a message. Such an inhibition of efficient translation may have resulted from the inability of the ribosomes to release the message once they had transversed the available sense codons. Termination usually proceeds via the action of peptidyl transferase and "release factors," whose activity depends upon a recognition event involving the nonsense codon (3, 8). The mRNA is released after the hydrolysis of the ester linkage between the tRNA and the carboxyl-terminal amino acid. The absence of a nonsense codon in RNAs transcribed from truncated DNAs resulted in a large reduction in translatability. For example, small t RNA transcribed from TaqI-cleaved DNA gave a 10-fold

smaller amount of translation of small t-related polypeptide relative to the amount of small t produced by an intact RNA. In addition, the shortened small t protein was reproducibly a broader band than that of full-length small t. These observations were probably the result of the accumulation of ribosomes at the 3' end of the RNA.

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