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Simian Virus 40 DNA Directs Synthesis of Authentic Viral Polypeptides in a Linked Transcription–Translation Cell-Free System

(*Escherichia coli* RNA polymerase/wheat germ extracts/protein analysis/restriction endonucleases/functional mapping of DNA)

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ABSTRACT **A linked cell-free system has been developed which is capable of transcribing and translating mammalian viral DNA, and its characteristics and requirements are outlined. In this system, simian virus 40 (SV40) DNA Form I (supercoiled) directed the synthesis of discrete polypeptides up to 85,000 daltons in size. One of these products was indistinguishable from authentic major virus capsid protein VP1, as judged by mobility on sodium dodecyl sulfate/polyacrylamide gels, antibody precipitation, and peptide analyses.**

The cell-free products larger than VP1 comprised a number of polypeptides ranging in molecular weight from 50,000 to 85,000. These polypeptides demonstrated no immunological relationship whatsoever to the structural protein VP1. However, two of these products, along with one of approximately 25,000 daltons, were precipitated with antiserum to SV40 tumor antigen. Linear SV40 DNA generated by the cleavage of Form I DNA with the restriction endonuclease *Eco*R$_I$ was an efficient template in this system and also directed the synthesis of a polypeptide migrating with VP1 on polyacrylamide gels. The potential of this system for defining a functional map of a DNA genome is discussed.

Simian virus 40 (SV40) is an oncogenic papova virus. Its DNA is a covalently closed circular molecule approximately 5500 nucleotide pairs in length, equivalent to a molecular weight of 3.6×10^6. The total potential genetic information contained within SV40 DNA is in the range of 200,000 daltons of protein and is divided about equally between functions expressed both early and late in the infection (1). Two viral structural proteins, VP1 (molecular weight 48,000) and VP3 (molecular weight 30,000), have already been characterized (2). RNA isolated from lytically infected cells late in infection directed the synthesis of the major capsid protein, VP1, in heterologous cell free systems (3, 4). In addition, indirect evidence has accumulated for the existence of a 70,000–100,000 dalton nonstructural viral protein, the tumor antigen (T-antigen) (5, 6).

Here we report the characteristics and requirements of an efficient linked cell free system utilizing *Escherichia coli* DNA-dependent RNA polymerase and wheat germ extracts. In this system, SV40 DNA directs the synthesis of discrete cell-free products up to 85,000 daltons in size. One of these polypeptides (molecular weight 48,000) is shown by a number of criteria to be indistinguishable from the major capsid protein, VP1.

On the basis of the accurate synthesis of VP1 and the appearance of other discrete products, the potential of this system for demonstrating the viral coding capacity, notably the synthesis of T-antigen, and for directly mapping DNA sequences that code for specific polypeptides is discussed.

Abbreviations: SV40, simian virus 40; T-antigen, tumor antigen; DNA FI, DNA Form I (supercoiled, closed circular); DNA FIII, DNA Form III (linear); VP, virus protein; $NaDodSO_4$, sodium dodecyl sulfate.

MATERIALS AND METHODS

Wheat germ was supplied by the Bar-Rav Mill, Tel-Aviv, Israel. [^{35}S]Methionine (240 Ci/mmol) was from Amersham and [^{3}H]UTP (20 Ci/mmol) from New England Nuclear Co. Phage T_4 DNA was a generous gift from P. J. Natale; hamster antiserum to SV40 T-antigen was purchased from Flow Labs. Rabbit anti-hamster IgG was from Microbiological Assoc.; additional samples of these sera were generously provided by D. M. Livingston; rabbit and goat antisera for immunoprecipitation of VP1 were kindly provided by H. Ozer. Restriction endonuclease *Bam*I from *Bacillus amyloliquefaciens H* was a generous gift from R. Roberts and *Hae*III from *Haemophilus aegyptius* from J. Newbold. *E. coli* K12 harvested in the middle of the logarithmic phase of growth was purchased from the Grain Processing Co., Muscatine, Iowa. *E. coli* DNA-dependent RNA polymerase was prepared by the method of Burgess wherein glycerol gradients are used (7). An additional low salt glycerol gradient was run to decrease the level of residual RNase activity.

Cells and Virus. A plaque-purified stock of SV40 (strain 777) was grown in the BSC-1 line of African green monkey kidney cells as described (8). [^{35}S]Methionine-labeled virus was prepared and purified (3).

The virus was disrupted by adding 0.4 volumes of a buffer containing 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl and 0.5% sodium dodecyl sulfate ($NaDodSO_4$). The solution was extracted with an equal volume of phenol followed by two extractions with chloroform/isoamyl alcohol (24:1). The interphase containing proteins was used as a source of virion proteins.

Closed circular viral DNA Form I (FI) was prepared from purified virions by extraction with phenol/chloroform/isoamyl alcohol (9) followed by centrifugation in a CsCl gradient supplemented with ethidium bromide (10). Ethidium bromide was removed by extraction with isopropanol saturated with CsCl. DNA was dialyzed against 10 mM Tris·HCl (pH 7.4), 1 mM EDTA and finally precipitated with ethanol in the

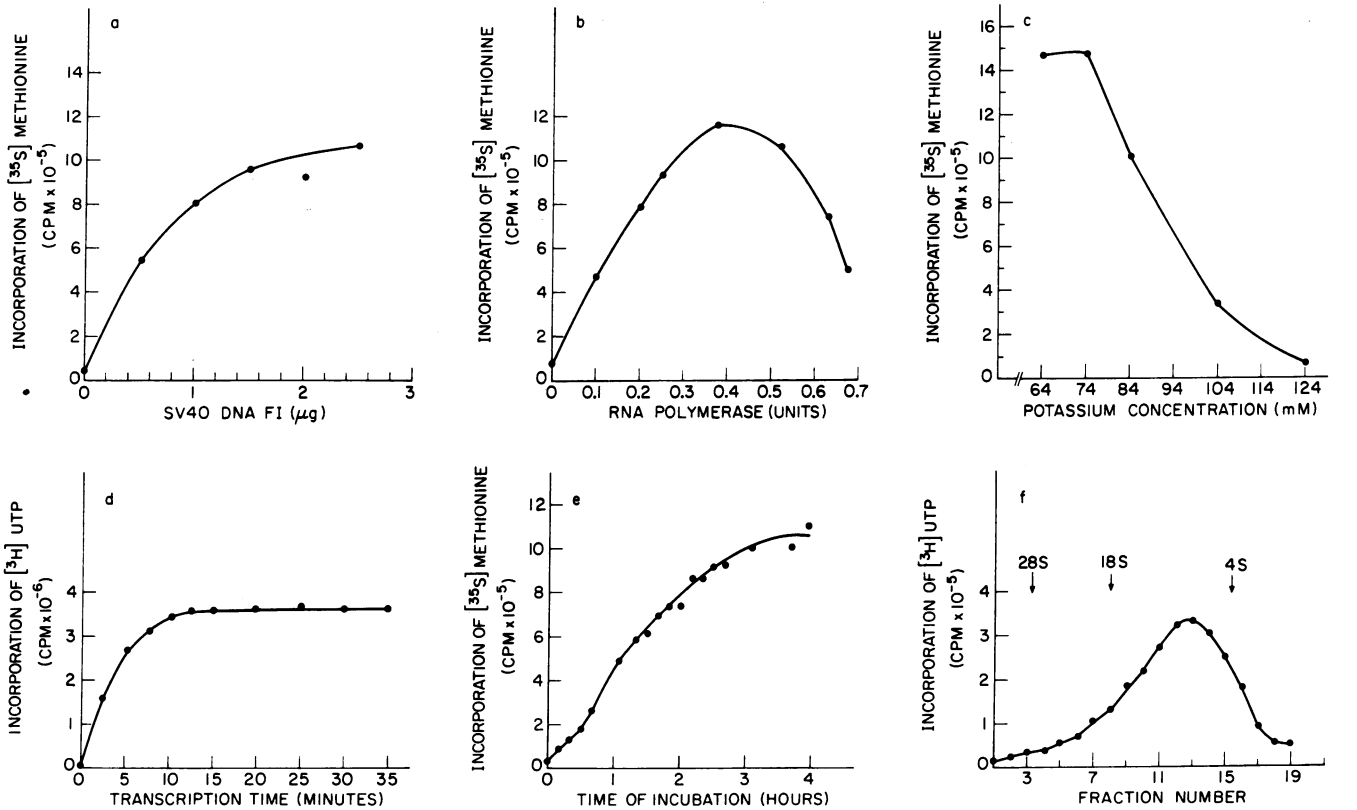

FIG. 1. Characteristics of the linked transcription-translation system. The conditions of the assays, unless otherwise specified, are as described in *Materials and Methods*. Stimulation of [^{35}S]methionine incorporation is shown as a function of concentration of (a) SV40 DNA FI, (b) RNA polymerase, and (c) KCl. Kinetics of incorporation are shown for (d) [^{3}H]UTP during transcription of SV40 DNA FI and (e) [^{35}S]methionine in the linked system. Fractionation of the [^{3}H]UTP-labeled RNA, transcribed from SV40 DNA FI in 15 min, on a 5–20% sucrose gradient containing 50% formamide and 0.2% $NaDodSO_4$, is shown in (f).

presence of 0.3 M NaCl. More than 95% of the DNA was in the form of supercoiled DNA as analyzed by mobility on agarose gels (11).

Digestion of SV40 DNA with Restriction Endonucleases. *Eco*R_1 enzyme was purified from *E. coli* strain RY 13 carrying the R_1 drug resistance plasmid (a generous gift from G. C. Fareed). The purification procedure and enzymic digestion of DNA were as described (12). A 1 μg sample of DNA was analyzed by electrophoresis in 0.6% agarose/10% glycerol gels and stained with ethidium bromide (0.5 μg/ml). Within the limits of detection (0.03 μg) the results indicated complete conversion of SV40 DNA FI into linear FIII. Digestion of SV40 DNA with *Haemophilus aegyptius* enzyme was performed as described (13). Electrophoretic analysis of the products indicated complete digestion.

RNA Fractionation and Hybridization. The labeled RNA synthesized during the transcription reaction was denatured and then fractionated in 5–20% sucrose gradients containing 50% formamide (14). Symmetry of the transcription was determined by self-annealing of the RNA followed by RNase digestion (15).

Linked Transcription-Translation. DNA-dependent RNA-directed protein synthesis was performed in two stages. A 10 μl reaction mixture was incubated for 15 min at 37° under conditions optimal for transcription. This was followed by a further 3 hr incubation at 23° after the addition of a 40 μl mixture that yielded final reaction conditions optimal for translation. The 10 μl transcription reaction contained 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (pH 7.9, adjusted with KOH), 10 mM magnesium acetate, 200 mM KCl, 200 μM spermine (free base), 0.5 mM each of ATP, GTP, CTP, and UTP, 5 mM dithiothreitol, 1–3 μg of SV40 DNA, and 0.37 units of purified *E. coli* RNA polymerase. After the addition of the 40 μl of translation mixture, the final 50 μl reaction contained 30 mM Hepes, (pH 7.0), 3 mM magnesium acetate, 75 mM KCl, 40 μM spermine, 100 μM GTP, 1 mM ATP, 8 mM phosphocreatine, 8 μg/ml of creatine kinase (155 units/mg), 1 mM dithiothreitol, 2 μM [^{35}S]methionine, 25 μM of the remaining 19 unlabeled amino acids and 10 μl of preincubated wheat germ extract prepared as described earlier (16).

Immunoprecipitation. VP-1 was immunoprecipitated using a modification of Tegtmeyer's procedure (17). Cell-free products (0.1 ml) or 50–125,000 cpm of ^{35}S-labeled $NaDodSO_4$-disrupted SV40 virions (0.1 ml) were incubated for 30 min at 37° with 0.01 ml of rabbit antiserum prepared against $NaDodSO_4$-treated virions. Goat antiserum to rabbit globulin (0.1 ml) was added, and the mixture was incubated for a further 30 min at 37° and then left overnight at 4°. The mixture was diluted to 3 ml with buffer [25 mM Tris·HCl (pH 7.4), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4] and centrifuged at 1000 × *g* for 20 min at 4°. The pellet was washed with 3 ml of cold buffer and recentrifuged. Cell free products were precipitated with hamster antiserum to SV40 T-antigen and rabbit anti-hamster IgG by the same procedure. The immuno-

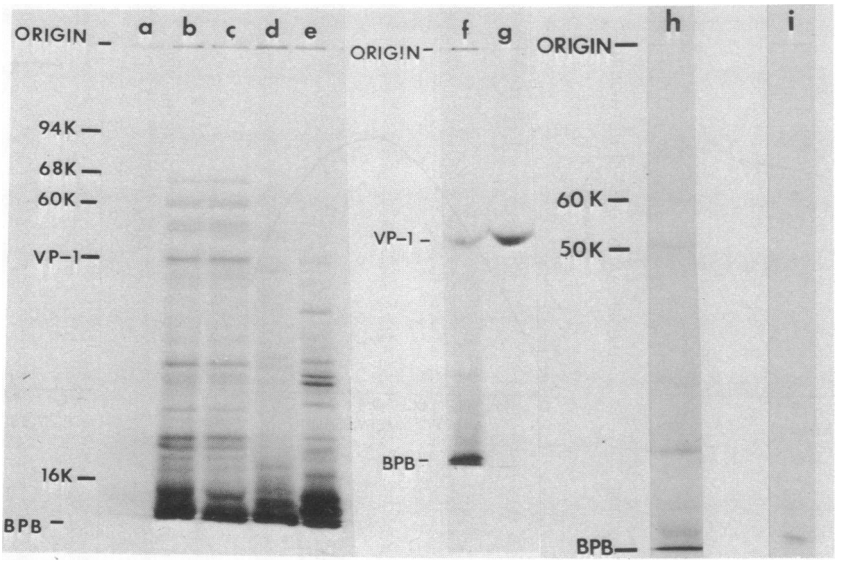

FIG. 2. Autoradiograms of [^{35}S]methionine-labeled polypeptides fractionated on NaDodSO$_4$/10–15% polyacrylamide gradient slab gels (3). Gels were stained with Coomassie blue, dried, and autoradiographed for 2 days. For a–e, the DNA template in the cell-free system (25 μl) was varied. (a) Control, without added DNA; (b) with 1 μg of SV40 DNA FI; (c) with 0.5 μg of SV40 DNA FIII, produced by cleavage of FI with *Eco*R$_1$; (d) with 1.5 μg of SV40 DNA digested with *Hae;* (e) with 0.5 μg of T4 DNA. Products immunoprecipitated as described in *Materials and Methods* are shown in f-i: (f) cell-free products directed by SV40 DNA FI precipitated using antiserum against disrupted SV40 virions; (g) authentic [^{35}S]methionine-labeled VP1 precipitated using antiserum against disrupted SV40 virions; (h) cell-free products directed by SV40 DNA FI precipitated using SV40 T-antigen antiserum from hamsters; (i) cell-free products treated with normal hamster serum. BPB indicates Bromophenol Blue. Standards of known molecular weight included phosphorylase a (94,000), bovine serum albumin (68,000), beef liver catalase (60,000), gamma globulin heavy chain (50,000), SV40 VP1 (48,000), and myoglobin (16,000).

precipitates were solubilized in 50 μl of electrophoresis buffer and 5–20 μl were analyzed on NaDodSO$_4$/polyacrylamide gels.

Peptide Analysis. The cell-free products were electrophoresed in preparative polyacrylamide slab gels, stained with Coomassie blue, and dried. The labeled polypeptide migrating with unlabeled VP1 was located by autoradiography. [^{35}S]-Methionine-labeled virus was fractionated by the same procedure. In each case the pieces of gel corresponding to VP1 were excised and 150 mg were rehydrated in 5 ml of 1% NH_4HCO_3, containing 50 μg/ml of L-1-tosylamido-2-phenylethylchloromethyl ketone treated trypsin (T. Morrison and H. F. Lodish, submitted for publication). After 16 hr incubation at 37°, the gel pieces were removed by centrifugation and the supernatant was lyophilized three times. The digests were fractionated on cellulose-coated plates.

RESULTS

The translation of transcribed RNA was carried out utilizing a modification of the wheat germ cell-free system. Several optimizations in the protein synthesis assay conditions resulted in a substantial increase in total efficiency. The reduction of pH from 7.6 to 7.0, the addition of 40 μM spermine (A. R. Hunter, R. T. Hunt, and R. J. Jackson, personal communication) and the reduction of creatine kinase concentration to 8 μg/ml resulted in a 5- to 10-fold increase in incorporation of [^{35}S]methionine over that reported previously (16). Under these optimized conditions, linear incorporation of amino acids continued for 2.5–3 hr. At least in the translation of tobacco mosaic virus RNA, the amount of "early quitting" of ribosomes was considerably reduced.

Characteristics of the Linked Cell-Free System. The incorporation of [^{35}S]methionine into trichloroacetic-acid-insoluble material was greatly enhanced upon addition of SV40 DNA FI. Stimulation was linear over a range of DNA concentration; a plateau was reached at 40 μg of DNA per ml of the complete reaction (Fig. 1a). The extent of incorporation was dependent upon the amount of *E. coli* RNA polymerase added during transcription and was characterized by an optimum DNA to polymerase ratio (Fig. 1b). Stimulation of incorporation was optimal at a potassium concentration of 70–75 mM; higher concentrations resulted in a reduction of the overall [^{35}S]-methionine incorporation (Fig. 1c). The transcription of SV40 DNA by *E. coli* RNA polymerase was linear for 10 min (Fig. 1d). Protein synthesis directed by this RNA after altering the reaction mixture for translation continued linearly for approximately 2.5 hr (Fig. 1e). On the average, the incorporation of [^{35}S]methionine in the presence of SV40 DNA was 80-fold higher than that in reactions incubated without DNA. The calculated efficiency of [^{35}S]methionine incorporation, 2–3 pmol/μg of DNA, is a conservative estimate of the total methionine incorporation. Isotope dilution experiments have demonstrated that the volume of wheat germ extract added to the final reaction contributes an unlabeled pool of 2–3 μM methionine (unpublished observation).

The requirements of the linked system are summarized in Table 1. They demonstrate that [^{35}S]methionine incorporation depended upon the addition of DNA, *E. coli* RNA polymerase, and nucleotides in the transcription reaction. Furthermore, incorporation was inhibited by the addition of 1 μg of DNase I (Worthington Biochemicals Co.) or 5 μg/ml of actinomycin D (Calbiochem Co.) at the beginning of the transcription reactions. Neither of these two constituents at

TABLE 1. *Requirements of the linked system*

Omission or addition	Incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble material	
	cpm × 10^{-4}	% of complete system
Complete system	190	100
− DNA; − polymerase	1.48	0.78
− Polymerase	4.10	2.16
− DNA	2.33	1.23
+ Actinomycin D (5 μg)	5.00	2.63
+ DNase (1 μg)	1.00	0.53
− Nucleotides	2.43	1.28

Reactions were in 50 μl volumes as described in *Materials and Methods*.

the concentrations used affected the translation of tobacco mosaic virus RNA in the orthodox wheat germ cell-free system (16).

The ^{3}H-labeled RNA transcribed from SV40 DNA FI by *E. coli* RNA polymerase was characterized by two methods. Denaturation and centrifugation in formamide sucrose gradients demonstrated the RNA to be heterogeneous in size, ranging from 4 S to 26 S with a peak at 10 S (Fig. 1f). Self-annealing of the total RNA synthesized showed that transcription was 10–20% symmetrical (i.e., from both of two complementary strands).

Nature of the Cell-Free Products. The ^{35}S-labeled cell-free products were fractionated according to molecular weights on Na Dod SO_4/polyacrylamide gradient slab gels and analyzed by radioautography. When DNA or polymerase or both were omitted from the reaction, the only polypeptide ever detected was one of 91,000 daltons (Fig. 2a). The complete linked system containing SV40 DNA synthesized a number of discrete polypeptides ranging from 14,000 to 85,000 daltons (Fig. 2b). Varying the final KCl concentration between 65 and 105 mM did not alter the spectrum of polypeptides synthesized (data not shown). A number of polypeptides of molecular weights 50–85,000 were clearly visible as well as a prominent polypeptide co-migrating precisely with VP1 marker (molecular weight 48,000). The products directed by SV40 DNA FIII generated by cleavage with the restriction endonuclease *Eco*R_1 were very similar to those synthesized under the direction of DNA FI. It is particularly noteworthy that a polypeptide co-migrating with marker VP1 was synthesized in apparent equal proportion with both templates (Fig. 2c). In contrast, this polypeptide was not synthesized when DNA cleaved with *Hae* enzyme was used as a template (Fig. 2d); *Hae* enzyme cleaves the entire late region into small fragments (18).

With phage T4 DNA as template, the spectrum of polypeptides synthesized was completely different from that directed by SV40 DNA (Fig. 2e).

Sequential treatment of the total cell-free products directed by SV40 DNA FI with rabbit antiserum prepared against NaDodSO_4-denatured virions and goat anti-rabbit IgG serum resulted in the specific precipitation of a [^{35}S]-methionine-labeled polypeptide (Fig. 2f) which migrated precisely with immunoprecipitated authentic ^{35}S-labeled VP1 (Fig. 2g). Similarly, treatment of cell-free products with SV40 T-antigen antiserum immunoprecipitated three polypeptides of approximate molecular weights 60,000; 50,000; and 25,000 (Fig. 2h). Clearly the two large polypeptides were not precipitated by serum against NaDodSO_4-disrupted virions (Fig. 2f) or normal hamster serum (Fig. 2i).

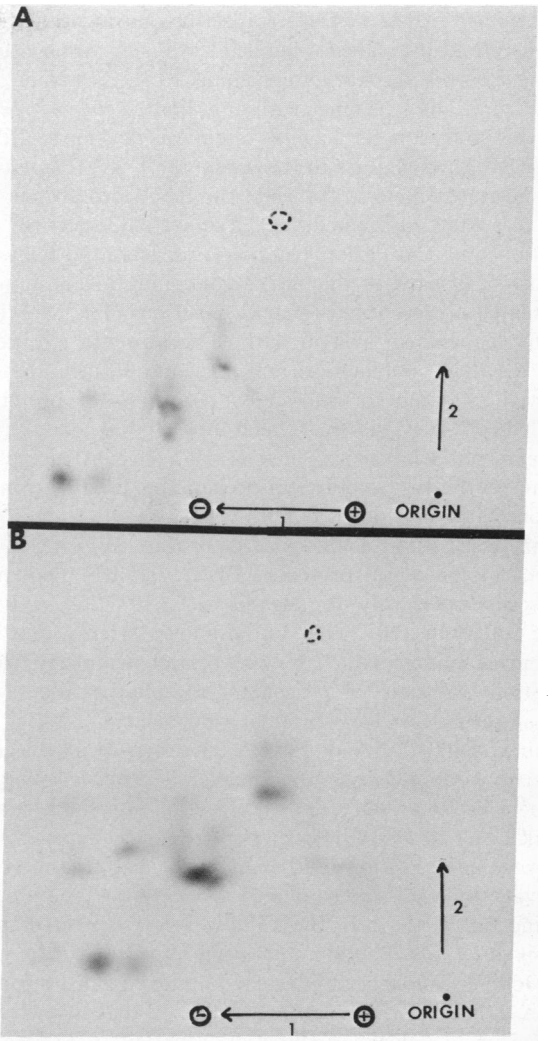

FIG. 3. Two-dimensional tryptic fingerprints of (A) putative VP1 synthesized *in vitro* and (B) authentic VP1 from purified virions. Samples prepared as described in *Materials and Methods* were subjected to ionophoresis at pH 3.5 at 900 V for 120 min followed by ascending chromatography in *sec*-butanol:propanol:isoamyl alcohol:pyridine:water (1:1:1:3:3). The broken circle denotes the position of ε-Dnp-lysine marker.

The [^{35}S]methionine-labeled tryptic peptides derived from electrophoretically purified authentic VP1 and the cell-free product migrating with VP1 were subjected to two-dimensional "fingerprint" analyses. Autoradiograms show that the *in vitro* (Fig. 3A) and *in vivo* (Fig. 3B) peptide patterns were identical except for one obvious extra peptide in the latter. Two-dimensional fractionation of the digests in other solvent systems confirmed these results (data not shown).

DISCUSSION

DNA-dependent cell-free systems derived from prokaryotes (19) have been shown to synthesize both bacterial and phage polypeptides as well as biologically active enzymes (20–22).

In most cases, these systems transcribe and translate simultaneously and are called "coupled" systems. Application of these exclusively prokaryotic systems to the study of mammalian viral DNA has met with only limited success (23, 24).

In this study a heterologous system was developed utilizing *E. coli* RNA polymerase to transcribe the DNA template and wheat germ extracts to translate the RNA into protein. This system is called a "linked" cell free system, since the transcription and translation reactions are separated temporally because of disparities among the potassium, magnesium, pH, and temperature optima for both reactions. The characteristics of the 'linked' system outline an absolute requirement for DNA, RNA polymerase, and nucleotides during the transcription reaction for subsequent synthesis of proteins. A coupled cell-free system, which operates at one-third the efficiency of the linked system, has also been constructed.

The synthesis of a polypeptide indistinguishable from VP1 demonstrates the fidelity of both transcription and translation in this system and confirms directly that SV40 DNA codes for the major capsid protein, VP1. *E. coli* RNA polymerase transcribes primarily the strand of SV40 DNA coding for early functions (15). The synthesis of a late protein in our system can be explained by the partial symmetry of transcription although the reason for the relative abundance of VP1 in the total cell-free product is not clear.

Linear SV40 DNA molecules generated by cleavage with restriction endonucleases function very efficiently as templates in the cell-free system. The introduction of a single staggered break in SV40 DNA by either restriction enzyme *Eco*R_1 (at cleavage map position zero) or *Bam*I (at map position 0.13) or cleavage by both was employed to locate the region of DNA coding for VP1. All of these linear DNA's directed the synthesis of a polypeptide migrating precisely with VP1 on $NaDodSO_4$/polyacrylamide gels (manuscript in preparation). RNA·DNA hybridization has indicated that late viral functions map in a clockwise direction from 0.65 to 0.17 map units (1). Since the size of the DNA from 0 to 0.17 map units is insufficient to code for VP1, we tentatively locate this function between 0.65 and 0 on the map. Our interpretation is based on the assumption that no extensive re-circularization of unit length DNA occurs during the cell-free reaction. After the transcription reaction, the DNA was determined to be still in a linear form by agarose gels, thus excluding only the possibility of ligation of the cohesive ends.

Other polypeptides synthesized in the cell-free system are not yet completely characterized. In particular there are discrete polypeptides specifically precipitated with SV40 T-antigen antisera, but not by antisera directed against structural polypeptides. These preliminary observations suggest the viral origin of T-antigen.

The availability of this cell-free system should facilitate the identification of exclusively virus-coded polypeptides, the direct mapping of viral DNA, the study of control mechanisms regulating the flow of information from DNA into polypeptides, and, in combination with hybridization techniques, the purification of specific eukaryotic genes. In the case of SV40 the use of restriction enzyme DNA fragments, as well as DNA from homogeneous defective populations, as templates in this cell-free system will facilitate the establishment of a functional map of the viral genome.

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