Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site

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ABSTRACT

The HSV gene encoding ICP4 is negatively regulated and the HSV gene encoding thymidine kinase is positively regulated by ICP4 in vivo. We report that ICP4 is a component of a stable complex that contains protein and a sequence of approximately 28 nucleotides that span the ICP4 gene transcription initiation site. The association of ICP4 with DNA sequences between positions -103 and +32 relative to the ICP4 mRNA start site was demonstrated by DNA binding immunoassays. DNase footprinting revealed that nucleotides between positions -8 and +20 are protected by ICP4. In contrast, binding of ICP4 to sequences flanking the mRNA start site in the thymidine kinase gene was not observed. Models for ICP4-mediated positive or negative regulation of HSV gene transcription are discussed.

INTRODUCTION

The genome of herpes simplex virus (HSV) contains approximately 70 genes. During the course of productive infection, transcription of viral genes is temporally and quantitatively regulated by cis-acting signals within the viral genome and by trans-acting factors encoded in both host and viral genes (1-11). The temporal pattern of expression for a given viral gene forms the basis for its classification as either an immediate early (alpha), early (beta), or late (gamma) gene (12).

Five genes have been assigned to the IE class. These genes encode infected cell polypeptides (ICPs) 4, 0, 22, 27 and 47, which have apparent molecular weights of 175,000 (175k), 110k, 68k, 63k, and 12k, respectively (13,14). The region that spans nucleotides approximately -100 to +40 relative to the transcription initiation site (+1) in each IE gene contains sufficient information to function as a constitutive RNA polymerase II promoter in uninfected cells (5,15). Each IE gene promoter is

regulated in a positive manner by cis-acting signals that are located in the region from -350 to -100 (4,5,16,17). This regulatory domain contains a binding site for a host cell protein (18) and is subject to positive regulation by a virion component designated as α -TIF or Vmw65 (4,8,19-21). When introduced by DNA transfection into uninfected cells, IE gene promoters function constitutively in the absence of viral proteins and are induced 10 to 40-fold in cells which contain α -TIF (8,22,23).

The expression of IE genes is also subject to negative regulation at several steps in the pathway from transcription to translation (24-27). The decreased production of ICP4 mRNA that occurs within 1 hour postinfection (28) implies that negative regulation is achieved in part by reducing the rate of IE gene transcription. Of particular interest is the observation that transcription from an ICP4 gene promoter domain that spans nucleotides -108 to +30 is reduced in cells which are cotransfected with the ICP4 gene (29). This suggests that ICP4 may play a role in negative regulation of IE gene expression.

The role of ICP4 in positive regulation of the early class of HSV genes at the transcriptional level has been well documented (26,30-32). However, attempts to define a cis-acting signal within the promoter-regulatory domain of an early gene which is required in vivo for induction by ICP4 have not been successful. All of the cis-acting domains which have been identified in the promoter-regulatory domain of the HSV-1 gene encoding thymidine kinase (tk) respond to proteins present in uninfected cells (1,33,34). Coen et al (1) have predicted that induction of tk gene transcription is not mediated by a direct interaction between ICP4 and the tk gene. In this paper we describe results which are consistent with this prediction.

Previous reports have established that stable protein-DNA complexes containing ICP4 can be formed in vitro (35-39). Based on results from mobility shift assays, Kristie and Roizman reported that there are binding sites for ICP4 in the promoter-regulatory domains associated with the IE genes encoding ICP4, 0, and 27 (36). Exonuclease III protection experiments revealed that the binding site in the ICP0 gene promoter is located between nucleotide positions -71 and -46 (37). Muller used a DMS

interference assay to map a binding site for ICP4 that spans nucleotides -10 to +3 relative to the ICP4 mRNA cap site (39). We have demonstrated by a DNA binding immunoassay that two regions within the procaryotic vector pBR322 and one region approximately 100 bp upstream of the HSV glycoprotein D transcription initiation site contain specific sequences which interact, directly or indirectly, with ICP4 (38). We have designated these sequences as ICP4/protein binding sites to indicate our inadequate knowledge regarding other proteins which may be required for formation of ICP4:DNA complexes under the conditions of this assay. Examination of the sequences protected in DNase I footprinting experiments revealed that the hexanucleotide sequence 5'-ATCGTC-3' is located at the same relative position in all three binding sites.

A computer search revealed that this hexanucleotide sequence is located in only two (namely, ICP4 and ICP0) of the four HSV-1 IE gene promoter-regulatory domains (the promoter-regulatory domains for the genes encoding ICP22 and ICP47 are identical). In this paper we report that when cloned fragments from each of the IE gene promoter-regulatory domains were used as target DNAs in DNA binding immunoassays, ICP4/protein binding sites were detected in the promoter domains of the genes encoding ICP0 and ICP4. No such sites were detected in the promoter-regulatory domains of genes encoding ICP22 and ICP27. DNase protection results revealed that the ICP4/protein binding site in the ICP4 gene promoter overlaps the ICP4 gene transcription initiation site, as shown previously by DMS interference (39). This location suggests that negative regulation of ICP4 gene expression may occur at the level of transcription initiation as a result of promoter occlusion by ICP4.

MATERIALS AND METHODS

Fraction VIII. Fraction VIII is the pooled peak of ICP4 that elutes from a DEAE-cellulose column in the purification scheme described previously (38,40). This partially-purified ICP4 was obtained from 1×10^{10} Vero cells infected with HSV-1 (HFEM).

Plasmid constructions. Plasmid pGX58 consists of the XhoI C fragment from HSV-1 DNA inserted into pMK16 (41). The SmaI/SalI

fragment spanning the ICP4 mRNA cap site was derived from pGX58 and inserted between the SalI and SmaI sites in pUC13 to construct pXK16. During construction of pXK16, two copies of a 17 bp SmaI/SalI vector fragment were inserted inadvertently at one junction between the vector and viral sequences. Consequently, the viral insert is flanked by SmaI sites.

The 1200 bp BamHI/SalI fragment that spans the ICP27 gene promoter (5,15) was obtained from plasmid pSG28 (42) and inserted between the BamHI and SalI sites in pUC13 to construct pXK81. The 762 bp BamHI/BglII fragment that spans the thymidine kinase (tk) gene promoter (43) was obtained by cleaving pHSV106 (Bethesda Research Labs) with BglII, filling in the sticky ends with T4 DNA polymerase, and then cleaving with BamHI. The BamHI end of the viral DNA fragment was ligated to the BamHI site in pMK16 and the blunt end was ligated to a blunt-ended (by T4 DNA polymerase) SalI site in pMK16 to construct pXK10. A BglII site was recreated as a result of the blunt end ligation.

DNA binding immunoassay. The DNA binding immunoassay was conducted as previously described (38). Plasmid DNAs were uniformly radiolabeled with ^{32}P in vivo so that all restriction fragments could be detected. Radiolabeled DNAs were digested with restriction enzymes and mixed with a 20-fold (by mass) ratio of unlabeled salmon sperm DNA in DNA binding buffer (DBB) (150 mM NaCl, 10 mM Tris·HCl (pH 7.5), 10 mM MgCl_2 , 2 mM mercaptoethanol, 0.5 mM EDTA, and 50 $\mu\text{g/ml}$ BSA). A sample of fraction VIII was added and the mixture was incubated for 30 min at 22°C. Polyclonal antibody that is specific for ICP4 (44) was added and allowed to react for 15 min at 22°C. The immune complexes were precipitated by the addition of protein A-bearing S. aureus. DNA fragments were eluted from the washed immunoprecipitate with buffer containing 2% NaDodSO₄, resolved by electrophoresis in 5% polyacrylamide gels, and detected by autoradiography.

RESULTS

Mapping high affinity ICP4/protein binding sites in HSV DNA. The DNA binding immunoassay described by McKay (45) is a useful technique for comparing the relative affinities of a site-specific DNA binding protein for different radiolabeled DNA targets under

circumstances where the protein is not available as a homogeneous preparation. When heterogeneous protein preparations are used in this assay, competing DNA is generally added to adsorb nonspecific

Figure 1. DNA binding assay with HSV-1 DNA fragments that contain the ICP4 gene promoter-regulatory region. Plasmid pXK16 contains a 508 bp DNA fragment from HSV-1 that extends from a SmaI site at -326 to a Sall site at +183 relative to the ICP4 mRNA start site (46). Uniformly-labeled pXK16 was digested with the indicated restriction endonucleases and used as target DNA with fraction VIII in DNA binding immunoassays. Panel A: Schematic diagram of a portion of pXK16 containing HSV (striped box) and vector (solid bar) DNA. Cleavage sites for PvuI (P), SmaI (S), EcoRI (E), and BamHI (B) are shown. Panel B: Autoradiogram showing DNA fragments immunoprecipitated with immune (lane I) IgG. pXK16 was cleaved with the restriction enzyme indicated over each pair of lanes and loaded into the corresponding lane M. The autoradiogram was trimmed so that large vector fragments do not appear.

DNA binding proteins. It should be noted that the competing DNA may also reduce binding of site-specific DNA binding proteins to "weak" binding sites in certain radiolabeled target DNAs. We have used the DNA binding immunoassay to investigate ICP4/protein binding sites in promoters from representative HSV genes. In general, these assays were performed under rather stringent conditions (i.e., in buffer containing 150 mM NaCl and a 20-fold ratio on a mass basis of salmon sperm DNA to target DNA) that favor the detection of target DNAs which have strong ICP4/protein binding sites.

Investigation of ICP4/protein binding sites in the ICP4 gene. A plasmid (pXK16) was constructed in which the 508 bp SmaI-SalI fragment extending from -326 to +182 relative to the ICP4 gene mRNA capsite (46) could be excised as a 525 bp SmaI fragment (Fig. 1A). When used as a target DNA, this 525 bp fragment was immunoprecipitated (Fig. 1B, lane 1). When the SmaI fragment was cleaved at position -103 with EcoRI, only the 302 bp EcoRI-SmaI fragment from position -103 to +182 was immunoprecipitated (Fig. 1B, lane 3). When the SmaI fragment was cleaved at position +32 with BamHI, only the 358 bp SmaI-BamHI fragment from position -326 to +32 was immunoprecipitated. When the SmaI fragment was cleaved at position +1 with PvuI, neither subfragment was precipitated. These results demonstrate that an ICP4/protein binding site is located between positions -103 and +32 in the ICP4 gene and suggest that nucleotides flanking the PvuI site at +1 are required for formation of stable protein:DNA complexes that contain ICP4.

The footprinting technique of Galas and Schmitz (47) was used to identify which nucleotides between positions -103 and +32 were protected by association with ICP4. A 332 bp fragment containing these nucleotides was labeled with ^{32}P at the 5' end of the EcoRI site (Fig. 1A), incubated with fraction VIII, and then briefly exposed to DNase I. The resultant radiolabeled DNA fragments were resolved by electrophoresis through a sequencing gel and detected by autoradiography. In the absence of fraction VIII, a ladder of bands was generated in which the intensity of the bands reflected the relative preference of DNase I for particular cleavage sites in the DNA (Fig. 2A). In the presence of fraction VIII, alterations in the pattern of DNase I cleavage were observed in

Figure 2. DNase I footprint of ICP4/protein binding site in the ICP4 gene promoter. Panel A: A 332 bp fragment (approximately 20 ng) extending from the EcoRI site at position -103 through the SalI site at +183 to the HindIII site in pXK29 was labeled at the 5' terminus of the EcoRI site with ^{32}P , incubated with fraction VIII (lanes 1-4: 0, 0.15, 0.45, or 1.35 μl , respectively), and then briefly treated with DNase I (lanes 1-4: 6, 6.4, 9.2, or 17.2 ng, respectively). Radiolabeled digestion products were separated by electrophoresis through an 8% polyacrylamide gel in the presence of 8.3 M urea and detected by autoradiography of the dried gel, as shown here. The numbers on the left and right sides of the gel indicate the position of the 3' end of the corresponding radiolabeled fragment, where position +1 corresponds to the ICP4 mRNA start site. Lanes G and A+G: DNA fragments produced by partial cleavage of the end-labeled fragments at guanines and at adenines plus guanines, respectively (48). Panel B: A schematic diagram of the region spanning the ICP4 gene promoter, showing restriction enzyme cleavage sites, the transcription start site, and the nucleotide sequence of the coding strand (46) from position -9 to +32. The position of the ATCGTC hexanucleotide is indicated by asterisks.

the region corresponding to positions -9 to +20 (Fig. 2A). In particular, increased cleavage by DNase I was observed near position -9 and decreased cleavage was observed near positions +2, +6, and +20. These results indicate that the region from -8 to

Figure 3. DNA binding assay with fragments spanning the ICP27 gene promoter. Plasmid pXK81 contains a 1200 bp fragment from HSV-1(KOS) extending from a BamHI site (B) at -272 to a SalI site (S) at +928 relative to the ICP27 mRNA start site. Uniformly labeled pXK81 was digested with BamHI and either SalI or HinfI (H) and used as target DNA in a DNA binding immunoassay with fraction VIII. Panel A: Schematic diagram of pXK81. HSV and vector DNAs are represented as in figure 1. Panel B: Autoradiogram of analytical gel. Either a BamHI-SalI or a BamHI-HinfI digest of pXK81 DNA was used as target DNA for the immunoassay (lane I). A sample from each digest was loaded into the marker (M) lanes.

+20 represents, at least in part, an ICP4/protein binding site. It is noteworthy that there is considerable overlap between the sequences (-8 to +20) protected from DNase digestion and the sequences (-10 to +3) where DMS modifications interfere with ICP4 binding (39).

Investigation of ICP4/protein binding sites in other IE genes.

The 1200 bp BamHI-SalI fragment between map units 0.740 and 0.748 in the HSV-1 genome extends from position -272 to +928 relative to the ICP27 gene transcription initiation site and contains both the regulatory and promoter domains for the ICP27 gene (15). A plasmid (pXK81) bearing this fragment was cleaved with BamHI and either SalI or HinfI and used as target DNA in a DNA binding immunoassay. With a long exposure, the 1200 bp BamHI-SalI fragment and the 326 bp BamHI-HinfI fragment that spans the ICP27 gene from -272 to +54 (Fig. 3A) were barely detectable in the DNA recovered from the immunoprecipitate (Fig. 3B). A vector (pUC13) that contains no strong ICP4/protein binding sites was chosen as the vehicle for pXK81 to ensure that there would be no competing strong ICP4/protein binding sites in this assay. The bands at 2730 bp and 1450 bp (Fig. 3B) are attributed to either very weak binding or nonspecific trapping of vector DNA. Thus, under the conditions of this assay, there are no high affinity ICP4/protein binding sites within the promoter-regulatory domains of the ICP27 gene.

Using a similar approach, we have observed a strong ICP4/protein binding site in the 77 bp BstNI fragment that extends from nucleotide -90 to -14 relative to the mRNA cap site in the ICP0 gene (49). We assume that this site corresponds to the region from -71 to -46 that was previously shown to contain an ICP4/protein binding site (37) and that includes the ATCGTC hexanucleotide sequence between -67 and -62 found during the computer search. We were unable to detect any strong binding sites in the promoter shared by the ICP22 and 47 genes using target DNAs that extended from -299 to -70, -97 to +7, or -57 to +201 relative to the mRNA cap site in this promoter (49). It should be noted that the fragment from -299 to -70 includes core sequences associated with the origin of DNA replication in the S component of the viral genome (50).

Investigation of ICP4/protein binding sites in the HSV-1 tk gene promoter-regulatory domains. The 762 bp BamHI-BglIII fragment between map units 0.30 and 0.31 in the HSV-1 genome extends from position -709 to +53 relative to the tk gene transcription initiation site (43). A plasmid (pXK10) bearing this fragment was cleaved with BamHI, PstI, HindIII, and XhoI and used as the target

Figure 4. DNA binding assay with fragments spanning the tk gene promoter. Plasmid pXK10 contains a 762 bp DNA fragment from HSV-1 that extends from a BamHI site at -709 to a BglIII site at +53 relative to the tk mRNA start site. Uniformly-labeled pXK10 was digested with a combination of HindIII (H), BamHI (B), PstI (P), and XhoI (X) and used as a target DNA in a DNA binding immunoassay with fraction VIII. Panel A: Schematic diagram of pXK10. HSV and vector DNAs are represented as in figure 1. The locations of ICP4/protein binding sites are indicated by asterisks (*). Panel B: Autoradiogram of analytical gel. Lanes 1 and 7: Samples from HindIII-BamHI-PstI-XhoI digest of pXK10 DNA; Lane 2: preimmune IgG; Lanes 3-6: Samples from binding reactions in which immune IgG was used, the mass of target DNA was fixed, and the mass ratio of carrier DNA (salmon sperm) to target DNA was either 0 (lane 3), 25 (lane 4), 50 (lane 5), or 100 (lane 6).

DNA in DNA binding immunoassays with fraction VIII. Of the six cleavage products (Fig. 4A), four are of particular interest, namely (i) a 490 bp fragment which extends from -709 to -220 relative to the tk gene, (ii) a 234 bp fragment which extends from -219 to +15 relative to the tk gene and contains the tk gene promoter-regulatory domains (1), (iii) a 346 bp fragment which contains the two strong ICP4/protein binding sites in the tet gene of the vector, and (iv) a 1702 bp fragment which contains a weak ICP4/protein binding site in the vector.

To ensure that any potential ICP4/protein binding sites in the viral DNA were not competed out with excess carrier DNA, a series of assays was performed in which the amount of target DNA was held constant and the mass ratio of carrier to target DNA was varied from 0 to 100. In the absence of competing DNA, the 1702 and 346 bp vector fragments were readily detectable in the immunoprecipitate (Fig. 4B), whereas the 490 bp fragment was barely detectable and the 234 bp fragment was not seen. Thus, under the conditions of this assay, there are no detectable

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AATGCGCTCATCGTCATCCTCGGCACCGTCACCCCTGGATGCTGTAGGCAT  pBR tet-1 (+98)
* !***** !!!** *!* ** *! ** *
TTGCGGGATATCGTCCATTCCGACAGCATCGCCAGTCACCTATGGCGTGCT  pBR tet-2 (+179)
* ! !***** ** * !** * ! ! * ! ! *
TGTGACACTATCGTCCATACCGACCCACCGACGAATCCCTAAGGGGGA  HSV-1 gD (-115)
!!* * * !***** !** * !* !* !* !* !*
GACGCCCCGATCGTCCACACGGAGCGCGCTGCCGACACGGATCCACGAC  HSV-1 ICP4 (-12)
!!* * !***** !** * !* !* !* !* !*
ATTGGGGGAATCGTCACCTGCCGCCCTTTGGGGAGGGGAAGGCGTGGGG  HSV-1 ICPO (-76)

nnYGnnnnnATCGTCnnYnCCGRCnnCRYCRnCRnRRnRYnnnRnnRnRRn  Consensus

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Figure 5. Comparison of nucleotide sequences spanning ICP4/protein binding sites. Sequences protected by ICP4 (and/or associated factors), as established in this and previous publications (37-39), are underlined and were aligned by matching the hexanucleotide sequence 5'-ATCGTC-3' present in each site. Asterisks (*) indicate perfect matches and exclamation marks (!) indicate pyrimidine-pyrimidine or purine-purine matches between a pair of sequences. For sites pBR tet-1 and tet-2, the number in parentheses indicates the position of the first nucleotide shown relative to the EcoRI cleavage site in pBR322. For the HSV-1 sites, the number in parentheses indicates the position of the first nucleotide shown relative to the transcription initiation site in the corresponding gene. The consensus sequence indicates that specific nucleotides (A, T, C, or G), purines (R), or pyrimidines (Y) occur at the same position in at least four of the five sequences shown. Positions where there is no apparent consensus are also indicated (n). The underlined portion of the consensus sequence corresponds to the maximum region that is protected in all five binding sites.

ICP4/protein binding sites within the sequences from -219 to +15 that define the known promoter-regulatory domains of the HSV-1 tk gene. The binding of ICP4 to the 490 bp fragment is extremely weak and has not been investigated further. Binding of ICP4 to a fragment from the tk gene that extends from nucleotide -709 to +53 was also extremely weak and binding to a fragment from +15 to +854 could not be detected (49).

Comparison of ICP4/protein binding sites. A comparison of the nucleotide sequences in ICP4/protein binding sites which have been identified (37-39) is shown in Figure 5. The similarity of portions of these sites is striking. For example, there is a 14 bp region in each of the ICP4, gD, and tet-2 gene sites in which there are 11 or 12 perfect matches, 1 pyrimidine match, and 1 or 2 complete mismatches. This 14 bp region includes the sequence 5'-ATCGTC-3', which is present in all ICP4/protein binding sites which we have identified, and thus presumably forms part of the recognition sequence required for association of ICP4 with DNA.

DISCUSSION

We report that ICP4 binds, either directly or indirectly, to sequences in the promoters of the immediate early HSV-1 genes encoding ICP4 and ICP0. These results confirm and extend the findings of Kristie and Roizman (36,37) and Muller (39). Muller demonstrated by DMS interference assays that the binding site in the ICP4 gene promoter includes nucleotides from position -10 to +3. The DNase protection results presented here suggest that the binding site may be somewhat larger, extending from -10 to +20. Alternatively, the protection of sequences downstream from +3 may be attributed to steric hindrance of the DNase by an "overhanging" portion of protein bound upstream of +3.

We also report that, under the conditions of the DNA binding immunoassay, the association of ICP4 with the promoter-regulatory domains of the IE HSV-1 genes encoding ICPs 22, 27, or 47 or with the regulatory domain of the ICP4 gene was extremely weak at best. These results differ from those of Kristie and Roizman (36,37), who reported that the regulatory domains of the ICP4 and ICP27 genes contain ICP4/protein binding sites. We note that these two domains lack the ATCGTC hexanucleotide sequence and speculate that

stable association of ICP4 with the regulatory domains of the ICP4 and ICP27 genes may require a factor which is present in nuclear extracts from infected cells (as prepared by Kristie and Roizman) but which is absent from more highly purified preparations of ICP4, such as fraction VIII used in this investigation.

As an extension of this idea, one could propose that ICP4 is an effector protein which can "dock" with a variety of DNA binding proteins that recognize different sequences in the promoter-regulatory domains of cellular and viral genes. Depending on the particular "docking" protein, the effect of this interaction could be either positive or negative. Thus, the interaction of ICP4 with cellular proteins that bind to early viral promoters (such as the tk gene) might increase promoter strength, whereas the interaction of ICP4 with factors that bind to immediate early viral promoters might decrease promoter strength. It should be noted that this model does not exclude the possibility that ICP4 itself is a sequence-specific DNA binding protein. Given the size of the polypeptide (1298 amino acids, ref. 51), it is not unreasonable to suggest that ICP4 has a domain for protein:DNA interactions as well as domains for protein:protein interactions.

The association of ICP4 with nucleotides spanning the ICP4 gene transcription initiation site is consistent with a model in which ICP4 represses initiation of transcription of the ICP4 gene by hindering the movement of RNA polymerase. The role of the ICP4/protein binding site in the regulation of ICP0 gene transcription is not as obvious. However, it may be more than coincidental that the ATCGTC hexanucleotide component of the ICP4/protein binding site at positions -66 to -61 is located immediately downstream from a CCAAT pentanucleotide at positions -75 to -71 that may bind the cellular transcription factor CTF/NF-1 (52) or CBP (53). Furthermore, the ICP4/protein binding site overlaps a GGGCGG hexanucleotide at positions -57 to -52 that may bind the cellular transcription factor Sp1 (6). It is also noteworthy that all three sites are apparently on the same face of the DNA helix. Thus, expression of the ICP4 gene may be exquisitely regulated in both a positive and a negative manner by multiple interactions among three different regulatory proteins that might bind to the ICP0 promoter within a 25 bp region (37).

In fact, Gelman and Silverstein (9) have reported that the ICP0 gene can be regulated either positively or negatively by ICP4, depending on the ratio of effector protein to target DNA.

We also report a direct demonstration that ICP4 itself does not bind with high affinity to the known promoter-regulatory domains of the HSV-1 thymidine kinase gene. Negative binding results were also obtained with a DNA fragment spanning nucleotides from -410 to +140 in the ICP4-inducible (11) promoter associated with the HSV-2 early gene encoding the 38k subunit of ribonucleotide reductase (49). These results support the proposal by Coen et al (1) that "protein products of HSV immediate early regulatory genes do not bind directly to an induction-specific domain of the tk promoter" and suggest that induction by ICP4 is mediated in an indirect manner. Experiments to test indirect mechanisms for ICP4-induced early gene expression are being conducted with in vitro transcription systems (35,54), which ultimately must be refined to their essential components before the definitive analyses can be performed.

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