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A cellular factor is required for transcription of vaccinia viral intermediate-stage genes

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ABSTRACT **The cytoplasmic location of vaccinia virus replication and evidence that the multisubunit DNA-dependent RNA polymerase, early and late stage transcription factors, capping and methylating enzymes, and poly(A) polymerase are virus encoded raised the possibility that all of the proteins needed for viral mRNA synthesis are of viral origin. Previous studies showed that four components from infected cells, the viral RNA polymerase and capping enzyme and two factors called vaccinia virus intermediate transcription factors (VITFs) 1 and 2, can reconstitute transcription of vaccinia virus intermediate-stage genes *in vitro*. Here, we demonstrate that VITF-2 can be isolated from the nuclei of uninfected HeLa cells as well as from the cytoplasm of infected cells. The proteins with VITF-2 activity from uninfected and infected cells cochromatographed and cosedimented, suggesting that they are identical. VITF-2 activity was found in extracts of other uninfected human and monkey cells but not in nonpermissive *Trichoplusia ni* insect cells or in conditionally permissive rabbit kidney 13 cells. VITF-2 activity was present, however, in a permissive line of rabbit kidney 13 cells that had been stably transfected with the vaccinia virus K1L host range gene. We suggest that the VITF-2 level acts as a gauge of the permissive state of the cell and thereby regulates the length of the early prereplicative phase of the infection.**

The poxviruses are a family of large, complex DNA viruses that replicate and express their genes in the cytoplasm of infected cells (1). Studies with vaccinia virus revealed the successive transcription of early, intermediate, and late classes of viral genes (2–5). Each gene class has a consensus promoter sequence that is recognized by cognate transcription factors (6–9). The enzymes and factors required for *in vitro* transcription of early genes are virus encoded and can be isolated from purified vaccinia virions (10). They consist of the multisubunit RNA polymerase (11–13), the RNA polymerase-associated protein RAP94 (14), the vaccinia virus early transcription factor VETF (15–17), and the capping enzyme (18–21). Factors required for intermediate transcription can be obtained from infected cells prior to replication of the vaccinia viral genome. They include the virus-encoded RNA polymerase and capping enzyme and an additional partially purified intermediate transcription factor (22–24). The viral RNA polymerase and at least two other virus-encoded proteins, vaccinia virus late transcription factors 1 and 2, are needed for *in vitro* transcription of late genes (4, 25–27).

Studies, such as the ones cited above, have led to suggestions that vaccinia virus transcription is carried out entirely by virus-encoded proteins. However, the intermediate and late transcription factors are not completely characterized. Recently, vaccinia virus intermediate transcription factors (VITFs) 1 and 2 were isolated from the cytoplasm of infected cells (48). In the present study, we demonstrate that VITF-2 also can be isolated from the nuclei of uninfected cells and suggest that this cellular protein may play a unique role in the regulation of vaccinia virus gene expression.

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MATERIALS AND METHODS

Virus and Cells. Human HeLa cells were grown in suspension culture in Eagle's medium supplemented with 5% (vol/vol) horse serum. Human 293, monkey CV-1, and rabbit RK13 and RK13-K1L cells were propagated in monolayer cultures in modified Eagle's medium containing 10% (vol/vol) fetal calf serum. Lepidopteran *Trichoplusia ni* cells were grown in SF-900 medium (GIBCO). The construction and properties of the RK13-K1L stably transfected cell line will be reported elsewhere (G.S., R.R., and B.M.). Procedures for the propagation of vaccinia virus have been described (28).

***In Vitro* Transcription.** The purification of vaccinia virus RNA polymerase, capping enzyme, VITF-1, and VITF-2 from HeLa cells infected with vaccinia virus in the presence of cytosine arabinoside has been described (48). Double-stranded DNA-cellulose column chromatography was the final purification step for RNA polymerase, VITF-1, and capping enzyme; VITF-2 was purified through the 0.4 M Q-Sepharose column chromatography step. Protein concentrations were determined by the method of Bradford (29).

Standard transcription reactions were carried out in 30-μl volumes containing 50 mM Tris·HCl (pH 8.0), 50 mM NaCl, 7.5% (vol/vol) glycerol, 0.15 mM EDTA, 5 mM $MgCl_2$, 150 ng of an uncleaved plasmid template containing the G8R promoter next to a DNA segment lacking guanylate residues in the nontemplate strand (G-less cassette), 1 mM ATP, 0.5 mM CTP, 0.05 mM UTP, 5 μCi (1 Ci = 37 GBq) of [α-^{32}P]UTP, 2 μl of RNA polymerase (0.1–0.2 mg/ml), 5 μl of capping enzyme (0.05–0.1 mg/ml), 5 μl of VITF-1 (0.1 mg/ml), and 5 μl of VITF-2 (0.5 mg/ml). Reactions were incubated for 30 min at 30°C and then for 10 min at 37°C in the presence of 500 units of RNase T1. Reactions were terminated by adding 0.25 ml of 150 mM NaCl and 0.3% SDS. The RNA was phenol/chloroform extracted and precipitated with ethanol in the presence of 0.2 μg of tRNA carrier. RNA was analyzed by electrophoresis in a 4% polyacrylamide/*N*,*N*'-methylenebisacrylamide (34:4) gel containing 8 M urea, followed by autoradiography.

Preparation of Nuclear and Cytoplasmic Fractions of HeLa Cells. Suspension cultures were centrifuged, and the cell pellets were resuspended in five volumes of buffer A [20 mM Tris·HCl, pH 7.9/20 mM KCl/1 mM dithiothreitol (DTT)/0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and allowed to

Abbreviations: VITF, vaccinia virus intermediate transcription factor; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

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swell in a Dounce homogenizer on ice while mixing gently every 5 min. After 20 min, the cells were broken with 20 (2–3 sec each) strokes of a B pestle. The homogenate was centrifuged at 4°C for 20 min at 10,000 rpm in a Sorvall S34 rotor. The supernatant was saved as the cytoplasmic fraction and treated as described below. The nuclei were washed with two volumes of buffer A and immediately centrifuged as before. The washed nuclei were suspended in two volumes of buffer B [20 mM Tris·HCl, pH 7.9/0.42 mM NaCl/1 mM $MgCl_2$/1 mM DTT/17% (vol/vol) glycerol/0.5 mM PMSF] and Dounce homogenized as already described. Particulate material was removed by centrifugation at 4°C for 30 min at 10,000 rpm. Solid ammonium sulfate (0.35 g/ml) was added to the supernatant, which was then stirred for 30 min on ice. After centrifugation at 4°C for 30 min at 10,000 rpm, the supernatant was discarded and the pellet was resuspended in buffer C [20 mM Tris·HCl, pH 7.9/1 mM DTT/1.5 mM $MgCl_2$/0.2 mM EDTA, 0.5 mM PMSF/25% (wt/vol) glycerol] containing 0.05 M KCl (using 3 ml for nuclei derived from 10 liters of cells). Pepstatin (5 μg/ml), aprotinin (5 μg/ml), leupeptin (20 μg/ml), antipain (5 μg/ml), and chymostatin (5 μg/ml) were added, and the mixture was dialyzed against 1 liter of buffer C containing 0.05 M KCl for 4 h at 4°C.

The cytoplasmic fraction was diluted with four original packed cell volumes of 50 mM Tris·HCl, pH 7.9/10 mM $MgCl_2$/2 mM DTT/25% sucrose/50% (vol/vol) glycerol and then mixed gently on ice for 30 min. Ammonium sulfate precipitation and centrifugation were carried out as described above, and the pellet was resuspended in buffer C containing 0.05 M KCl and protease inhibitors (30 ml for cytoplasm derived from 10 liters of cells) and dialyzed.

Chromatography of HeLa Cell Extracts. Cytoplasmic or whole HeLa cell extracts were prepared as described (30, 31). The ammonium sulfate precipitate was dissolved in buffer C containing 0.05 M KCl and protease inhibitors and dialyzed as above. Approximately 40 ml of dialyzed cytoplasmic extracts of vaccinia virus-infected HeLa cells (5 mg/ml) or whole-cell extracts of uninfected HeLa cells (5 mg/ml) was applied to 20-ml DEAE-cellulose columns and washed with three column volumes of buffer C containing 0.05 M KCl. The bound proteins were eluted with a discontinuous gradient of 0.15, 0.5, and 1.0 M KCl in buffer C. The 0.5 M KCl fraction (30 ml at 0.2 mg/ml) containing VITF-2 activity was dialyzed against 0.05 M KCl in buffer C and applied to a 20-ml heparin-agarose column. The flow-through containing VITF-2 was then applied to a 15-ml Q-Sepharose column that was washed with three column volumes of 0.05 M KCl in buffer C. Proteins were eluted using a 90-ml linear 0.05–1.0 M KCl gradient in buffer C. Fractions (3 ml) were collected, and the KCl concentrations were determined by conductivity.

Glycerol Gradient Sedimentation. Pooled Q-Sepharose column fractions (14–18) were dialyzed against buffer D [50 mM Tris·HCl, pH 8.0/1 mM DTT/0.2 mM EDTA/0.1 M KCl/10% (vol/vol) glycerol/0.5 mM PMSF] and applied to a linear 10–30% glycerol gradient on top of a 60% cushion in buffer D. Centrifugation was at 45,000 rpm at 4°C for 24 h in a SW50.1 rotor. Fractions of 0.2 ml were collected from the top of the gradient.

RESULTS

Detection of VITF-2 Activity in Nuclear Extracts of Uninfected HeLa Cells. We recently isolated two intermediate transcription factors, VITF-1 and VITF-2, from the cytoplasm of HeLa cells infected with vaccinia virus in the presence of cytosine arabinoside, an inhibitor of DNA replication (48). The two factors, together with the viral RNA polymerase and capping enzyme, were necessary and sufficient to reconstitute transcription of intermediate-promoter regulated templates *in vitro*. Neither VITF-1 nor VITF-2 could be replaced by cytoplasmic extracts of uninfected cells, suggesting that they were viral proteins. This prediction was confirmed for VITF-1, which was purified to homogeneity and shown to be encoded by the E4L open reading frame of the vaccinia virus genome (48). Our most highly purified preparations of VITF-2, however, contained several polypeptides, and amino acid sequence analyses of tryptic peptides of some did not correspond to any open reading frame in the vaccinia virus genome (unpublished data). We decided, therefore, to further examine the possibility that VITF-2 is a cellular protein.

A comparison of lanes 1 and 2 in Fig. 1 shows that VITF-2 is absolutely required, in addition to the other three components, for transcription of a template containing the G8R intermediate gene promoter. Unexpectedly, extracts of whole, uninfected HeLa cells were able to substitute for purified VITF-2 (Fig. 1, lanes 3–5) but not for any of the other transcription components (lanes 6–8). These data suggested that VITF-2 might be a host protein or that a host protein could substitute for VITF-2. A difference between this experiment and our initial one cited above was that whole-cell extracts of uninfected cells were used instead of the cytoplasmic fraction. To test whether this was the critical factor, the nuclear and cytoplasmic fractions of uninfected and infected HeLa cells were prepared and assayed. This experiment demonstrated that VITF-2 activity was present almost exclusively in the nuclear fraction of uninfected cells, whereas it was present in both nuclear and cytoplasmic fractions of infected cells (Fig. 2).

Cochromatography and Cosedimentation of VITF-2 from Infected and Uninfected Cells. Further experiments were needed to determine whether the VITF-2 activities of infected and uninfected cells could be attributed to the same protein. Accordingly, proteins from cytoplasmic extracts of infected cells and whole extracts of uninfected cells were subjected to parallel purification schemes. The VITF-2 activities from both sources bound to DEAE-cellulose columns and were eluted stepwise with 0.5 M KCl. After dialysis, the activities that flowed through heparin-agarose columns were bound to Q-Sepharose columns. Upon application of a linear gradient, both activities were eluted between 0.4 and 0.5 M KCl (Fig. 3). The peak VITF-2 fractions were then applied to

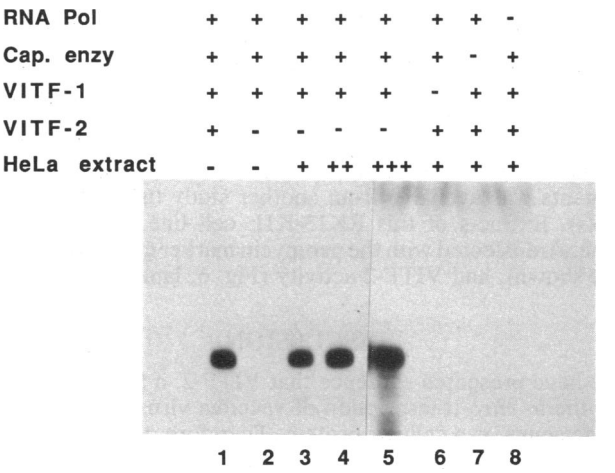

FIG. 1. VITF-2 activity in extracts of uninfected HeLa cells. Standard transcription reactions using a template with a G8R intermediate promoter were carried out with (+) or without (−) the indicated components in a total volume of 30 μl as described in *Materials and Methods* except for addition of 2.5 (+), 25 (++), or 50 (+++) μg of HeLa cell extract. RNA was analyzed by polyacrylamide gel electrophoresis, and an autoradiogram is shown. Pol, polymerase; Cap. enzy, Capping enzyme.

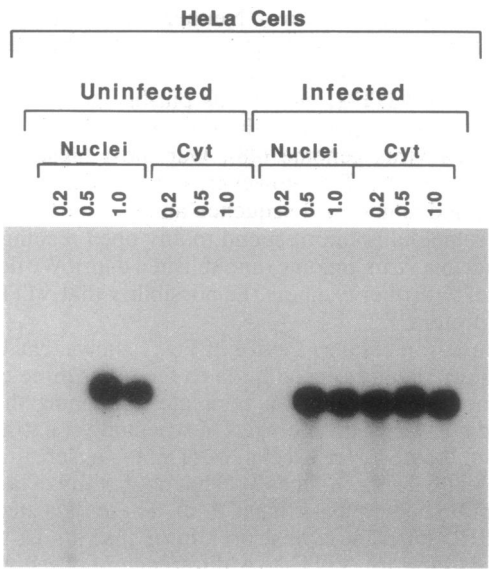

FIG. 2. Subcellular location of VITF-2 activity in uninfected and vaccinia virus-infected HeLa cells. HeLa cells were disrupted by Dounce homogenization, and nuclei and cytoplasmic fractions were separated by centrifugation. Standard transcription assays contained the indicated volume (0.2–1.0 μl) of nuclear or cytoplasmic (Cyt) extract (5 mg/ml) from uninfected or vaccinia virus-infected HeLa cells in place of VITF-2. RNA was analyzed by polyacrylamide gel electrophoresis, and an autoradiogram is shown.

linear 10–30% (vol/vol) glycerol gradients where they sedimented at approximately the position of the 68-kDa marker (Fig. 4). The similar chromatographic and sedimentation properties suggested that the same protein is responsible for VITF-2 activity in infected and uninfected cells.

VITF-2 Activity in Other Cell Lines. Since vaccinia virus has a wide host range, we assumed that VITF-2 activity is present in most mammalian and avian cells. Indeed, VITF-2 activity was detected in whole-cell extracts of uninfected human 293 and monkey CV-1 cells, although activity in HeLa cells was consistently highest (Fig. 5, lanes 3, 5, and 6). Vaccinia virus has not been reported to replicate in insect cells, so that the absence of VITF-2 activity in extracts of *T. ni* lepidopteran cells was not surprising (Fig. 5, lane 7). More intriguing was the absence of detectable VITF-2 activity in extracts of rabbit kidney RK13 cells (Fig. 5, lane 4). Although wild-type vaccinia virus replicates well in RK13 cells, viral host range mutants with an inactive K1L gene undergo abortive infection (32, 33). An RK13 cell line that was stably transfected with the K1L gene regulated by a simian virus 40 promoter and is permissive for vaccinia virus K1L deletion mutants was available from another study (unpublished results). Extracts of this RK13-K1L cell line, but not from others transfected with the neomycin marker gene alone (data not shown), had VITF-2 activity (Fig. 6, lanes 5 and 6).

DISCUSSION

We have presented evidence that VITF-2, a factor required for the *in vitro* transcription of vaccinia virus intermediate-stage genes, is a cellular protein. Therefore, the supposition that vaccinia virus encodes all of the enzymes and factors required for transcription of its genome may be incorrect. Supporting *in vivo* experiments, however, await the identification of the cellular protein. While it seems unlikely that cellular factors are needed for transcription of early stage genes, the factors for late transcription have been insufficiently characterized to make a similar prediction.

Vaccinia virus DNA and RNA appear to be synthesized exclusively in the cytoplasm of infected cells (34). Thus, the

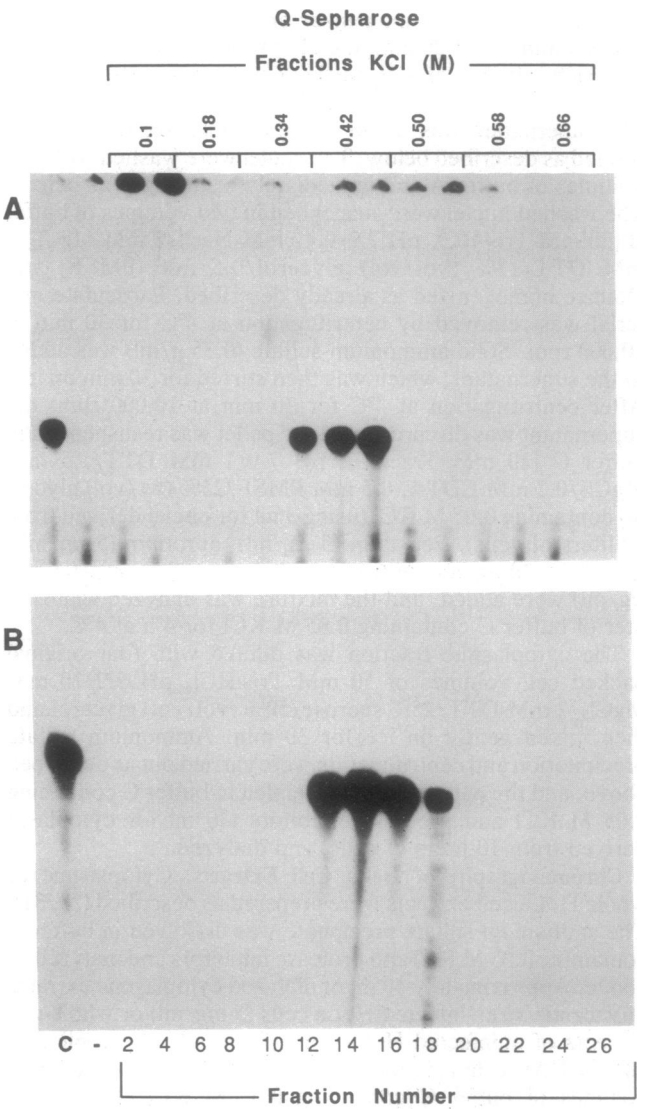

FIG. 3. Cochromatography of VITF-2 from uninfected and infected cells. Cytoplasmic extracts of vaccinia virus-infected HeLa cells (*A*) and whole-cell extracts from uninfected HeLa cells (*B*) were chromatographed on DEAE-cellulose, heparin-agarose, and Q-Sepharose. Aliquots (10 μl) from the Q-Sepharose column fractions were assayed in standard transcription reactions lacking VITF-2. Lane C contains all necessary transcription components including VITF-2, and lane (−) lacks VITF-2. RNA was analyzed by polyacrylamide gel electrophoresis, and autoradiograms are shown.

apparent translocation of VITF-2 from the nucleus to the cytoplasm after infection is consistent with the latter site for intermediate transcription. In this regard, previous studies have suggested that nuclear RNA polymerase II translocates to the cytoplasm after poxvirus infection (35–37), although the evidence that this enzyme has a role in vaccinia viral gene expression is indirect (36–39). The presence of VITF-2 in the cytoplasm of infected cells could also result from the activation of a resident cryptic form of the protein. Such a model is reminiscent of the inactive cytoplasmic complex of NF-κB and I-κB (40, 41). After induction with phorbol ester, the dissociated NF-κB is transported to the nucleus where it participates in cellular gene expression. By contrast, activated VITF-2 would remain cytoplasmic. A third possibility is that vaccinia virus recruits newly synthesized cytoplasmic VITF-2 before it reaches the nucleus. Distinguishing between these possibilities would be facilitated by the acquisition of reagents including VITF-2-specific antibodies and nucleic acid probes. Enucleation experiments, which previously

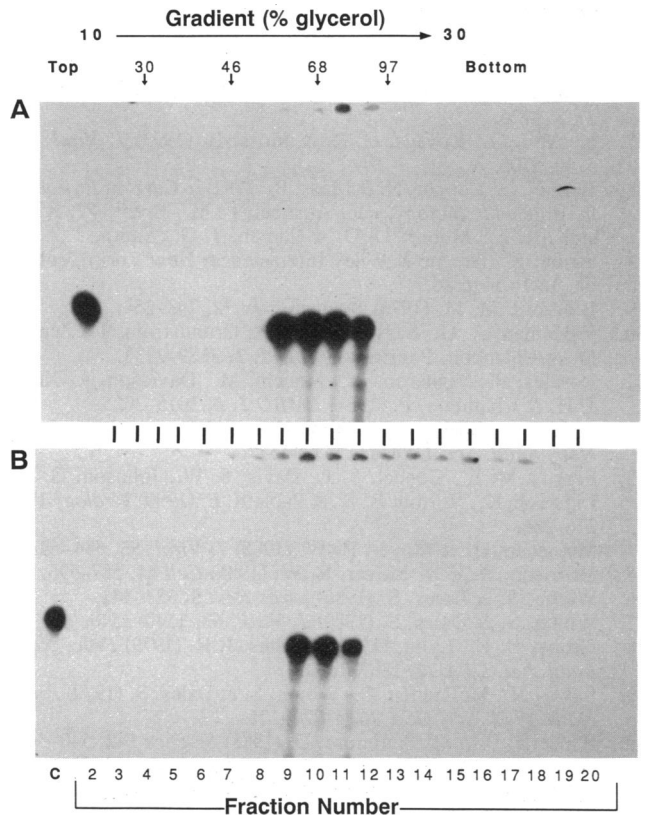

FIG. 4. Glycerol gradient sedimentation of VITF-2 from infected (*A*) and uninfected (*B*) cells. Pooled fractions from the Q-Sepharose column in Fig. 3 were sedimented through linear 10–30% (vol/vol) glycerol gradients on top of 60% cushions. Aliquots (10 μl) were assayed in standard transcription reactions lacking VITF-2. Lane C contains all necessary transcription components including VITF-2. RNA was analyzed by polyacrylamide gel electrophoresis and autoradiography. Aliquots from parallel gradients containing marker proteins were analyzed by SDS/PAGE. The positions and masses (kDa) of the marker proteins are indicated at the top.

indicated a role for the nucleus at a late stage in vaccinia virus replication (42–44), might provide useful information.

Vaccinia virus can replicate in a wide range of mammalian and avian cells. Therefore, if VITF-2 is essential, it must be a highly conserved protein. In addition to HeLa cells, we found VITF-2 activity in human 293 and monkey CV-1 cells but not in nonpermissive insect cells. The absence of VITF-2 activity in rabbit kidney RK13 cells was particularly interesting because this cell line is conditionally permissive. Wild-type vaccinia virus can replicate to high titers in RK13 cells, but virus mutants lacking a functional K1L gene exhibit a host range defect and are blocked at an early stage (33, 45). Nevertheless, the K1L mutants still replicate in other cells, and the role of the K1L protein, which contains ankyrin repeats (46), is unknown. Viral intermediate transcription is blocked in RK13 cells infected with K1L deletion mutants, but DNA replication is also affected, suggesting a very early or pleiotropic effect (G.S. and A. Ramsey-Ewing, unpublished results). We could detect VITF-2 activity in a RK13 cell line that stably expresses the viral K1L gene and is permissive for vaccinia virus K1L deletion mutants. Further studies are needed, however, to determine whether VITF-2 activity is linked to K1L expression.

Why should vaccinia virus depend on a cell factor for intermediate transcription when it encodes a multisubunit RNA polymerase, other transcription factors, and numerous enzymes? Perhaps the active VITF-2 level is a gauge of the permissive state of the cell and thereby regulates the length

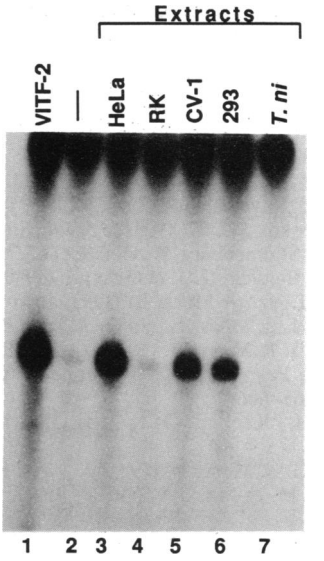

FIG. 5. Comparison of VITF-2 activities in whole-cell extracts prepared from various cell lines. Extracts were prepared and assayed as in Fig. 1 using RNA polymerase, capping enzyme, VITF-1, and either purified VITF-2 (lane 1), no VITF-2 (lane 2), 0.2 μl of HeLa extract (lane 3), or 2 μl of extracts from RK13 (RK; lane 4), CV-1 (lane 5), 293 (lane 6), or *T. ni* (lane 7) cells. RNA was analyzed by polyacrylamide gel electrophoresis and autoradiography.

of the early prereplicative phase of the infection. Thus, in cells that have active VITF-2, transition to the intermediate and late stages of infection would occur rapidly. In cells that have low levels of VITF-2, viral early proteins such as the vaccinia virus epidermal growth factor homolog (47) and the K1L protein would have a prolonged period to upregulate a variety of host functions needed for replication. By contrast, a completely programmed transcription system composed exclusively of viral factors would not be attuned to the intracellular environment and could cause premature and irreversible entry into the intermediate and late phases of the infection cycle.

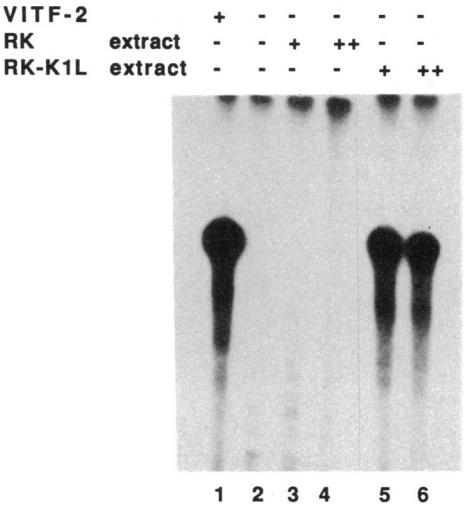

FIG. 6. VITF-2 activity in RK13 cells stably transfected with the vaccinia virus K1L gene. Standard transcription assays were run using RNA polymerase, capping enzyme, VITF-1, and either VITF-2 (lane 1), no VITF-2 (lane 2), 5 (+) or 10 (++) μl of extracts from RK13 (RK; lanes 3 and 4), or RK13-K1L (RK-K1L; lanes 5 and 6). RNA was analyzed by polyacrylamide gel electrophoresis, and an overexposed autoradiogram is shown.

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