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Expression of the three influenza virus polymerase proteins in a single cell allows growth complementation of viral mutants

(temperature-sensitive mutants/bovine papilloma virus vector/protein synthesis/single-gene expression)

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ABSTRACT **Transformed cell lines derived from murine C127 cells were constructed that express the influenza virus RNA-dependent RNA polymerase proteins (PA, PB1, and PB2). Cell lines that express only one or all three of the proteins were tested for their ability to complement temperature-sensitive viral mutants incubated at the nonpermissive temperature. Two cell lines were isolated that express all three polymerase genes and complement the growth of PB2 temperature-sensitive mutants at the nonpermissive temperature. One of these lines also complemented PA temperature-sensitive mutants. The viral titers obtained in these two cell lines were 12-fold to 1000-fold higher than the viral titers obtained upon growth of the corresponding temperature-sensitive mutant in C127 cells at the nonpermissive temperature.**

The influenza A virus genome is composed of eight single-stranded RNA segments of negative polarity. Each of the three largest RNA segments codes for one of the different RNA-directed RNA polymerase molecules (PB2, PB1, and PA) that, along with the nucleoprotein and virion RNA, comprise the virion nucleocapsid (1, 2). The functions of the polymerase proteins with regard to RNA transcriptase activity have been partially defined (2). The initial step of transcription is the binding of polymerase to capped mRNAs, which are used to generate primers for viral mRNA. This step is carried out by the PB2 protein (3–5). Crosslinking experiments have also shown that the PB1 protein is associated with initiation of transcription and chain elongation (3, 5). No function has as yet been ascribed to the PA protein, although during transcription a complex composed of the three polymerase proteins moves down the elongating mRNA (28).

One of our primary goals has been to develop a complementation system for influenza viruses through the use of mammalian cells engineered to express one or more of the influenza virus polypeptides. The expressed viral protein may be active during subsequent viral infection. This type of cell system can be useful for the functional analysis of protein domains and may allow the isolation and/or growth of viral mutants. The catalytic nature of the polymerase proteins makes them good candidates for use in a complementation system, as large amounts of expressed protein may not be needed to exert their effects.

In developing a complementation system, it is desirable to use a continuous cell line. For this reason we have used a bovine papilloma virus (BPV) vector along with the mouse metallothionein-1 (*Mt-1*) promoter to construct our cell lines (6). Recent studies have shown that BPV vectors can be used to construct cell lines expressing various influenza virus proteins from C127 or NIH 3T3 murine cells (7–9). In this paper, we discuss cell lines constructed to express one or all of the three polymerase proteins. These cell lines were tested for their ability to complement known temperature-sensitive (ts) mutants of influenza virus. These ts mutants have proven useful for genetic studies, although their inherent properties of "leakiness" and reversion have made the assignment of functions to specific viral proteins difficult (10). Two assays were used to determine complementation activity. The first assay detects viral protein synthesis in mutant infected cells incubated at the nonpermissive temperature. All polymerase-expressing cell lines showed some activity in this assay, although to varying degrees. The second complementation assay measures yield of infectious virus in mutant-infected cells incubated at the nonpermissive temperature. In this assay, only cell lines expressing all three polymerase proteins test positive with certain groups of ts mutants. One cell line (3P-133), when infected with PB2 ts mutants at the nonpermissive temperature, exhibits up to a 1000-fold increase over ts virus yields obtained from C127 cells at the nonpermissive temperature.

MATERIALS AND METHODS

Plasmids, Cells, and Viruses. Plasmids pBMT3X and pSPR1 are described in *Results*. Plasmids pAPR101, pAPR206, and pAPR303 have been described (11). C127 cells (C127I) were obtained from the American Type Culture Collection (CRL 1616). Transformed cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 μM $CdCl_2$. A/WSN/33 (ts^+) virus and ts mutants ts1 (PB2 mutant), ts15 (PB1 mutant), and ts53 (PA mutant) were grown in MDBK or MDCK cells as described (12–15).

Isolation of Transformed Cells. C127 cells were transfected with 10 μg of vector DNA by a modification of the calcium phosphate precipitation procedure (16). One day after transfection, cells were split 1:20, and 5 μM $CdCl_2$ was added 12–24 hr later. Medium was changed every 3–4 days and $CdCl_2$ concentration was increased stepwise to 20 μM. Individual cell foci were picked at 2 weeks and expanded.

Complementation Assays. In the protein synthesis assay, cells were infected with WSN ts^+ or ts mutants for 1 hr at 33°C. Medium was then added and cells were shifted to 39.5°C. Infected cells were labeled metabolically at 6–7 hr postinfection (hpi) with [^{35}S]methionine and examined by $NaDodSO_4$/7–14% PAGE as described (17, 18).

For titration of infectious virus (plaque-forming units, pfu), cells were infected at 33°C for 1 hr (multiplicity of infection, 10). Cells were washed three times with phosphate-buffered saline (29), medium containing 0.1% trypsin was added, and cells were incubated at the nonpermissive temperature (39.5°C). Medium was harvested at 24 hpi and virus was titrated at the

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Abbreviations: BPV, bovine papilloma virus; hpi, hr postinfection; pfu, plaque-forming unit(s); ts, temperature-sensitive.

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permissive temperature (33°C) to assay total infectious virus produced. In some experiments, antibody to WSN virus was added for 1 hr at 2–3 hpi to remove any residual infecting virus.

Dot Blot Analysis of Cell Lines. Total cellular RNA was extracted from cells grown in the presence of 10 μM $CdCl_2$, using guanidinium thiocyanate (19). Dot blot hybridization was performed with a vacuum filtration manifold (Schleicher & Schuell) as described (20), using 3-fold serial dilutions of cellular RNA. Nick-translated probes made from purified polymerase gene cDNA were used for blot-hybridization experiments.

RESULTS

Construction of Vectors. The BPV vector used in these experiments is shown in Fig. 1*A*. The plasmid pBMT3X (G.P., unpublished work) is a shuttle vector that contains the entire BPV-1 genome, as well as pML2 (30) sequences that are necessary for transformation of and replication in bacterial cells. This vector also contains the human metallothionein-1A gene (21), which can be used as a selectable marker because it confers to C127 cells resistance to increased levels of heavy metals such as cadmium. Also included in this vector is the mouse *Mt-1* gene (22) that contains an engineered *Xho* I site preceding the initiation codon for the Mt-1 protein. This *Xho* I site is used as the insertion site for expression of influenza virus genes under the control of the mouse *Mt-1* promoter. Polymerase cDNAs were inserted either directly into pBMT3X or first into pSPR1 (Fig. 1*B*). The final constructions containing the PB1, PB2, and PA genes were designated pBMT3X/PB2, pBMT3X/PB1 and pBMT3X/PA, respectively (Fig. 1*A*).

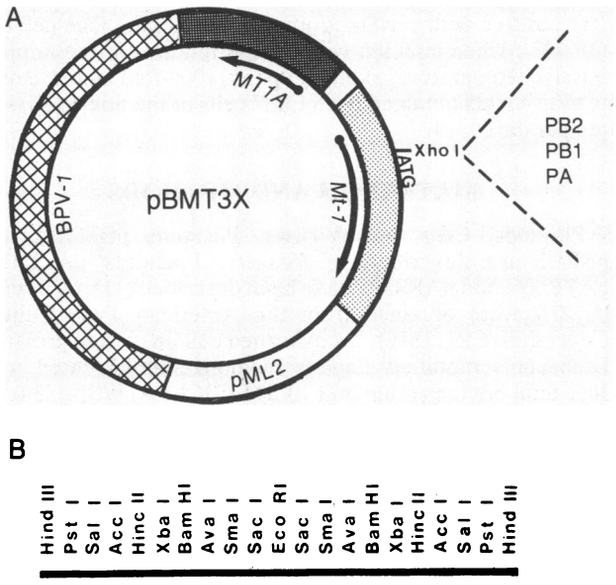

FIG. 1. Construction of pBMT3X/PB2, pBMT3X/PB1 and pBMT3X/PA. (*A*) Plasmid pBMT3X. The BPV-1, human metallothionein-1A (*MT-1A*), murine metallothionein-1 (*Mt-1*), and pML2 regions in pBMT3X are indicated. The directions of transcription from the metallothionein promoters in the human (*MT1A*) and murine (*Mt-1*) metallothionein genes are depicted by arrows. The single *Xho* I site in the *Mt-1* gene precedes the initiation codon of the murine Mt-1 protein. (*B*) Linker sequence of plasmid pSPR1. Plasmid pSPR1 was constructed by mixing plasmids pSP64 and pSP65 (27) (obtained from Promega Biotec, Madison, WI), which are similar except that the orientation of the linker sequence is reversed. After digestion with *Pvu* I and *Eco*RI, the mixture was religated and used to transform competent *Escherichia coli* MM294 cells. The resulting plasmid, pSPR1, contains an inverted terminal repeat of a linker sequence so that the *Eco*RI site is bounded on both sides by an additional 10 restriction sites. The PB2 and PA genes (11) were first inserted into plasmid pSPR1 at the *Bam*HI and *Eco*RI sites, respectively. Both genes were excised from pSPR1 by *Sal* I digestion, and purified inserts were inserted into the *Xho* I site of pBMT3X. The PB1 cDNA insert of pAPR 206 (11) was inserted directly into pBMT3X through end-filling of the terminal *Hin*dIII sites used in the initial cloning and addition of *Xho* I linkers (CCTCGAGG).

Analysis of Cell Lines Expressing a Single Polymerase Protein. C127 cells were transfected in three separate experiments with each of the three polymerase expression vectors (pBMT3X/PB2, pBMT3X/PB1, and pBMT3X/PA) and $CdCl_2$-resistant foci were isolated. Cell lines from each of the three transfection experiments were selected and used for further study. Cell lines PB2-5, PB1-1, and PA-18 were shown to express significant levels of PB2, PB1, and PA RNAs, respectively (see below). Due to the lack of high-titer antisera against the three polymerase proteins, we have not been able to directly detect expression of polymerase proteins in these lines. However, if these cell lines express polymerase proteins, it is possible they may function during subsequent influenza viral infections and complement viral mutants. Thus, these three cell lines were tested for complementation activity by examining protein synthesis of mutant-infected cells. WSN influenza virus mutants with known ts defects in the PB2 (ts1), PB1 (ts15), and PA (ts53) polymerase proteins were used to infect cell lines PB2-5, PB1-1, and PA-18, respectively. Viral protein synthesis after incubation for 6 hr at the nonpermissive temperature was examined (Fig. 2). Little or no virus-specific protein synthesis was detected in C127 cells infected with any of the three ts mutants (lanes 4, 7 and 10). However, the PB2-5 cell line infected with ts1 virus (lane 5) produces nearly wild-type levels of virus-specific proteins (lane 2). Similarly, the ts15-infected PB1-1 cells (lane 8) and the ts53-infected PA-18 cells (lane 11) showed increased viral protein synthesis over control (lanes 7 and 10, respectively). In ts^+-infected PB2-5, PB1-1, and PA-53 cells (lanes 3, 6, and 9), there was also enhanced viral protein synthesis compared to the control C127 cells (lane 2).

That these cell lines exhibit complementation activity with regard to viral protein synthesis indicates that the polymerase protein expressed from the cDNA can at least partially overcome a block caused by the mutant-coded polymerase. We next tested whether these cell lines exhibit complementation of infectious virus production by ts mutants at the nonpermissive temperature. The PB2-5, PB1-1, and PA-18 cell lines were infected with ts mutants and incubated at 39.5°C. C127 cells and cell line 3X-3, a cell line constructed by transforming C127 cells with the parental pBMT3X plasmid, were used as controls. Medium was harvested and titrated at 33°C in MDCK cells to determine total pfu production. The results are outlined in Table 1. PB2-5 cells infected with ts1 yielded, on average, a 5.6-fold higher titer than that obtained from ts1-infected C127 cells. Cell line PB1-1 produced viral yields only 1.7 times the control level when infected with the PB1 viral mutant ts15, whereas cell line PA-18 produced yields only 27% of control values of ts53-infected C127 cells. The pfu production from the 3X-3 control line was equivalent to that obtained from C127 cells using either ts^+ or the ts mutants. Clearly, all the cell lines expressing specific influenza virus genes show little or no complementation in an infectious-virus assay.

Construction of Cell Lines Expressing All Three Polymerase Proteins. Recent work (23) has indicated that C127 cells can harbor as extrachromosomal elements more than one type of BPV genome. Therefore, we transfected equal mixtures of the three BPV polymerase-cDNA-containing vectors into C127 cells in an attempt to obtain expression of all three polymerase genes in a single cell. Two separate transfection experiments were done and $CdCl_2$-resistant foci were selected. All cell lines selected were able to enhance viral protein

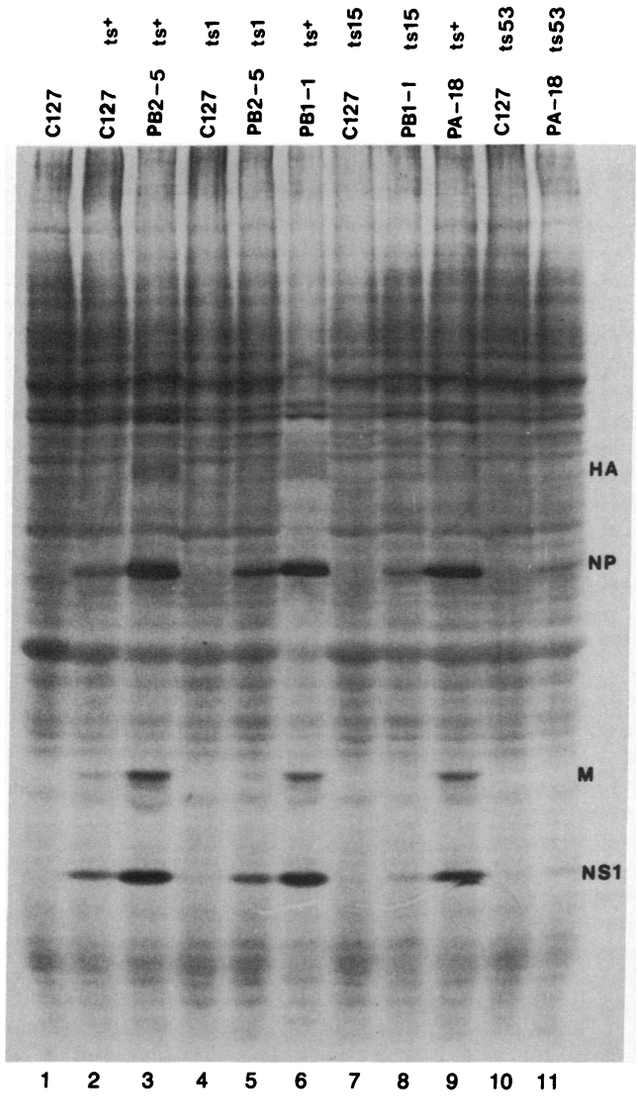

FIG. 2. NaDodSO$_4$/7–14% PAGE analysis of infected cells that continuously express a single polymerase gene. Infected cells were incubated at 39.5°C and labeled as described (18). The cell line used and the infecting virus are shown at the top of each lane (cells represented in lane 1 were mock-infected). The virus-encoded HA, NP, M, and NS1 proteins are indicated.

synthesis by ts mutants at the nonpermissive temperature. Two cell lines (3P-38 and 3P-133) were selected for further analysis. The results with cell line 3P-133 are shown in Fig. 3. Virus-specific polypeptides are seen in 3P-133 cells infected with ts1 (lane 6), ts15 (lane 8), and ts53 (lane 10), whereas virus-specific proteins are not apparent in C127 cells infected with these mutants (lanes 5, 7, and 9). Therefore, cells that express all three virus-specific polymerase genes exhibit complementation activity with all three groups of polymerase protein mutants. However, in all experiments the level of complementation seen in ts15-infected cells was much less than that seen in cells infected with ts1 or ts53.

In contrast to cell lines expressing individual polymerase genes, cell lines expressing all three polymerase genes (3P-38 and 3P-133) were able to complement the growth of ts mutants at nonpermissive temperature. The results of 5–10 experiments are summarized in Table 2. Both cell lines produced significantly higher titers upon ts1 infection than the control cell lines. 3P-38 cells produced 42-fold higher titers of ts1 than did infected C127 cells, while 3P-133 cells infected with ts1 gave 326-fold higher titers than control cells.

Table 1. Complementation of ts influenza virus mutants in transformed cell lines

Cell line	Titer, pfu/ml			
	ts$^+$	ts1	ts15	ts53
C127	6.1×10^7	4.3×10^3	3.7×10^3	2.8×10^5
3X-3*	4.9×10^7	8.8×10^3	—	9.7×10^4
	(0.80)	(2.0)		(0.35)
PB2-5	8.7×10^6	2.4×10^4	—	—
	(0.14)	(5.6)		
PB1-1	2.0×10^7	—	6.3×10^3	—
	(0.33)		(1.7)	
PA-18	2.0×10^7	—	—	7.7×10^4
	(0.33)			(0.27)

Data represent average titers (pfu/ml) obtained from 2–10 experiments. Values in parentheses indicate ratios of titer in transformed vs. C127 cells.

*The control cell line 3X-3 was constructed by transforming C127 cells with the parental pBMT3X plasmid.

Complementation titers obtained in some experiments with 3P-133 cells were as high as 1000-fold over control. Therefore, both 3P-38 and 3P-133 cells can significantly complement the growth of the PB2 mutant ts1 at the nonpermissive temperature. These cell lines also exhibited complementation activity with another PB2 mutant, ts6 (data not shown). Further analysis revealed that virus obtained as a result of complementation at the nonpermissive temperature remains ts in character.

Cell line 3P-38 also complemented the PA mutant ts53. The viral infection yielded a 12-fold higher titer than that obtained from the infected C127 controls. Cell line 3P-133 showed little growth complementation for ts53 virus, as yields were only 3.6-fold higher than control values. Neither cell line significantly complemented growth of the PB1 mutant ts15. Infected 3P-38 cells produced a yield only slightly higher than did infected C127 cells (3-fold), whereas the yield from infected 3P-133 cells was slightly lower than control.

RNA Analysis. It is clear that cell lines 3P-38 and 3P-133 can complement ts mutants with different efficiencies. In an effort to understand the differing characteristics of these two cell lines, we examined the relative steady-state levels of virus-specific RNAs in these cells. Total RNA from transformed cell lines was extracted and quantitated through dot blot hybridization. Presumably, the majority of the polymerase-specific RNA is present as mRNA. The results are shown in Fig. 4. 3P-133 cells contained relatively high levels of PB2-specific RNA and lower levels of PA- and PB1-specific RNA, whereas 3P-38 cells contained higher levels of PA and PB1 RNA than PB2-specific RNA. Using virion RNA from A/PR/8/34 virus as a standard, we estimate that the approximate steady-state level of polymerase-specific (PB2, PA, and PB1) RNA is around 300 copies per cell for the highest expressing cell line in each set. Note that the level of PB2-specific RNA in cell line PB2-5 is relatively high compared to that seen in cell line 3P-38, although in the pfu-production assay, 3P-38 was better than PB2-5 in complementing ts1.

DISCUSSION

In this paper we report the construction of cell lines that can complement the growth of ts mutants of influenza virus at the nonpermissive temperature. Specifically, we have used C127 cell lines transformed with BPV vectors designed to express the three influenza virus polymerase proteins. We first examined cell lines (PB2-5, PB1-1, and PA-18) each expressing only one polymerase gene in a protein complementation assay (Fig. 2). It has recently been shown that NIH 3T3 cells

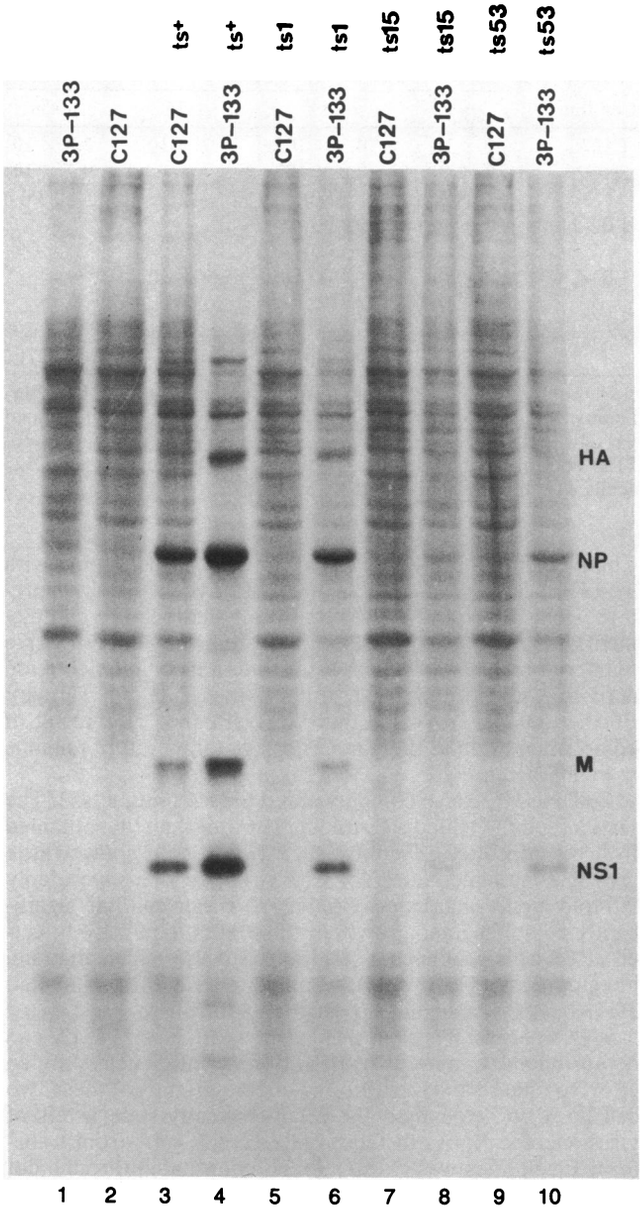

FIG. 3. NaDodSO$_4$/7–14% PAGE analysis of virus-infected cells (3P-133) that continuously express the three polymerase genes. The cells and infecting virus are indicated above each lane (for lanes 1 and 2, cells were mock-infected). Influenza virus-specific polypeptides are labeled.

expressing the PB2 protein from A/PR/8/34 virus can complement viral mRNA and protein synthesis of ts6, a PB2 mutant (9). We also observe this complementation phenotype with our C127 cells that express the PB2 gene. In addition, complementation of protein synthesis was observed in cells expressing either the PB1 or PA gene. The level of viral protein synthesis in ts1-infected PB2-5 cells is always higher than that in ts15-infected PB1-1 cells or in ts53-infected PA-18 cells and is, in fact, nearly equivalent to that of ts$^+$-infected C127 cells (Fig. 2, lanes 2 and 5). These results suggest that any of the three polymerase proteins expressed in the C127 cells can be incorporated into polymerase complexes and can be active during viral infection. However, none of the three cell lines expressing a single polymerase gene was able to complement significantly the growth of its corresponding ts mutant. This result is similar to that reported for the 3T3 cell line expressing the PB2 gene (9). Transfection of a mixture of

Table 2. Complementation of ts influenza virus mutants in cell lines expressing three viral polymerase genes

Cell lines	Titer, pfu/ml			
	ts+	ts1	ts15	ts53
C127	6.1×10^7	4.3×10^3	3.7×10^3	2.8×10^5
3P-38	4.8×10^7	1.8×10^5	1.1×10^4	3.3×10^6
	(0.79)	(42)	(3.0)	(12)
3P-133	4.8×10^7	1.4×10^6	3.3×10^3	1.0×10^6
	(0.79)	(326)	(0.89)	(3.6)

Data are average titers (pfu/ml) obtained in 5–10 separate experiments. Values in parentheses indicate ratios of titer in transformed vs. C127 cells.

the three vectors resulted in the isolation of cell lines expressing three viral polymerase genes (Fig. 4). Two cell lines, 3P-38 and 3P-133, were tested for their ability to complement ts mutants belonging to either the PB2, PB1, or PA groups. When infected with the ts mutants at the nonpermissive temperature, both cell lines were able to support viral protein synthesis at 6 hpi.

Although a protein synthesis complementation test could provide a functional *in vivo* assay for polymerase gene activity, of prime importance to us is whether these transformed cell lines can actually complement the growth of viral mutants. Such a system may allow for the isolation of new polymerase gene mutants of influenza virus, including deletion mutants. Significant growth complementation, as measured by pfu production at 39.5°C, is accomplished in ts1 (PB2 mutant)-infected 3P-38 or 3P-133 cells and in 3P-38 cells infected with ts53 (PA mutant). The PB1 mutant was not complemented significantly in this assay. This complementation value correlates well with the observed levels of viral protein synthesis in ts15- or ts53-infected 3P cells. Similar complementation phenotypes were observed when alternative ts mutants were used in these experiments (PB2 mutant ts6; PB1 mutant ts101; and PA mutant ts4).

The extent of growth complementation for each ts mutant varied for the two 3P cell lines (Table 2). These differences in complementation phenotype led us to examine the level of expression of the viral polymerase RNAs (Fig. 4). 3P-133 and 3P-38 cells contained different ratios of the polymerase RNAs. 3P-133 cells, which efficiently complement the PB-2 mutant, express a relatively high level of PB2 RNA and less PA or PB1 RNA. The 3P-38 cell lines, on the other hand, expresses higher levels of PB1 and PA RNA and is more effective for complementation of ts53 than 3P-133 cells. Therefore, the differences in complementation phenotype between the two cell lines could be a direct result of the different steady-state levels of the polymerase mRNAs.

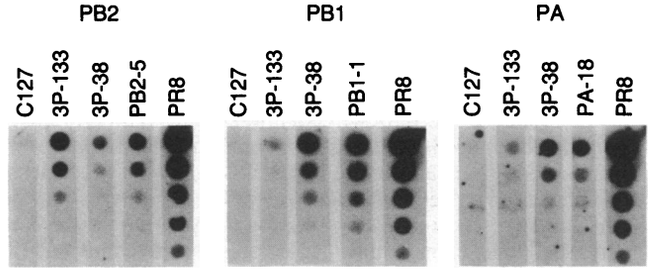

FIG. 4. Dot blot analysis of RNA levels in transformed cell lines. The nick-translated probe used in the experiments is indicated above each set. Cell lines used are indicated above each lane. Five 3-fold dilutions of each RNA preparation were spotted, beginning with a total amount of 5 μg of RNA. Lanes marked PR8 contain an RNA preparation from C127 cells to which 10 μg of virion RNA was added. Again, a series of five 3-fold dilutions was used for this control.

Presumably, cell lines expressing different ratios of the three polymerase proteins could be constructed that may improve the complementation of the PB2, PA, or PB1 mutants.

To determine complementation titers, the yields obtained from infections at 39.5°C were titrated in MDCK cells at the permissive temperature of 33°C. Since the cDNA gene used to express the polymerase protein was constructed from A/PR/8/34 virus (11), it is possible that marker rescue occurs through the replacement of the ts gene of the mutant with the PR8 gene. Although viral yields at the nonpermissive temperature of 39.5°C did not suggest that rescue occurred at high frequency, we cannot rule out the possibility that this type of genetic reassortment does occur in the system.

Clearly, the presence in a single cell of all three polymerase proteins produces a synergistic effect upon growth complementation compared to the cell lines expressing only one of the genes. There are a number of possible explanations for this synergistic complementation effect. It is possible that the relative levels of polymerase proteins may differ considerably from the relative levels of mRNA. For example, in the 3P-38 and 3P-133 cell lines, polymerase complexes may be formed that are resistant to degradation by host-cell proteases. The proteins made in the lines that express a single polymerase gene may be more susceptible to protease attack. In this context, it has been shown that PB2 protein expressed in NIH 3T3 cells has a half-life of only about 3 hr (9). Another explanation is that the polymerase proteins need to be complexed in order to function effectively. In the 3P lines, wild-type complexes may be formed prior to infection, whereas in the single-protein-expressing lines the polymerase polypeptide must be incorporated into complexes during infection. Therefore, there would be more active polymerase complexes available early in infection in the 3P-38 and 3P-133 lines. However, neither of these explanations can reconcile the fact that the level of viral protein synthesis detected in ts1-infected PB2-5 cells is similar to the level of protein synthesis in ts^+-infected C127 cells (Fig. 2). This has also been observed in ts6-infected NIH 3T3 cells expressing PB2 (9). So although protein synthesis in ts1-infected PB2-5 cells approaches wild-type levels, growth complementation is not observed. In order to explain this result, we favor a preliminary working model such that the polymerase complexes combine to form a multimeric complex, such as a trimer. If a multimeric complex contains one ts polymerase protein in its inactive configuration, it may not be stable for use in the production of mature virions. Therefore, in the ts1-infected PB2-5 cell, if half the polymerase complexes contain the inactive ts protein, then only one-eighth of the "trimers" of polymerase complexes would contain complexes without ts protein. In the 3P lines, these trimers, which may form prior to ts infection, are stable and can be packaged into virions. Therefore, in ts1-infected PB2-5 cells there may not be enough "wild-type" multimeric complexes to signifcantly increase the production of viable virus.

In summary, we report the engineered expression of viral genes in cell lines that can complement the growth of a negative-strand RNA virus. It is our hope that eventually such cell lines can be used to propagate mutants of influenza virus as has been achieved for many of the DNA viruses, such as simian virus 40, adenovirus, and herpesviruses (24–26).

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1. Lamb, R. A. (1983) in *Genetics of Influenza Viruses*, eds. Palese, P. & Kingsbury, D. W. (Springer, New York), pp. 21–69.
2. Krug, R. M. (1983) in *Genetics of Influenza Viruses*, eds. Palese, P. & Kingsbury, D. W. (Springer, New York), pp. 70–98.
3. Ulmanen, I., Broni, B. A. & Krug, R. M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7355–7359.
4. Blass, D., Patzelt, E. & Kuechler, E. (1982) *Nucleic Acids Res.* **10**, 4803–4812.
5. Ulmanen, I., Broni, B. A. & Krug, R. M. (1983) *J. Virol.* **45**, 27–35.
6. Pavlakis, G. N. & Hamer, D. H. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 397–401.
7. Lin, B.-C., Lai, C.-J. & Law, M. F. (1984) in *Segmented Negative Strand Viruses*, eds. Compans, R. W. & Bishop, D. H. L. (Academic, London), pp. 167–172.
8. Sambrook, J., Rogers, L., White, J. & Gething, M. J. (1985) *EMBO J.* **4**, 91–103.
9. Braam-Markson, J., Jeudon, C. & Krug, R. N. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4326–4330.
10. Mahy, B. W. J. (1983) in *Genetics of Influenza Viruses*, eds. Palese, P. & Kingsbury, D. W. (Springer, New York), pp. 192–254.
11. Young, J. F., Desselberger, U., Graves, P., Palese, P., Shatzman, A. & Rosenberg, M. (1983) in *The Origin of Pandemic Influenza Viruses*, ed. Laver, W. G. (Elsevier, New York), pp. 129–138.
12. Sugiura, A., Tobita, K. & Kilbourne, E. D. (1972) *J. Virol.* **10**, 639–647.
13. Sugiura, A., Ueda, M., Tobita, K. & Enomoto, C. (1975) *Virology* **65**, 363–373.
14. Krug, R. M., Ueda, M. & Palese, P. (1975) *J. Virol.* **16**, 790–796.
15. Palese, P., Ritchey, M. B. & Schulman, J. L. (1977) *J. Virol.* **21**, 1187–1195.
16. Parker, B. A. & Stark, G. R. (1979) *J. Virol.* **31**, 360–369.
17. Ritchey, M. B., Palese, P. & Schulman, J. L. (1976) *J. Virol.* **20**, 307–313.
18. Young, J. F. & Palese, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6547–6551.
19. Glisin, V., Crkvenjekov, R. & Byus, C. (1974) *Biochemistry* **13**, 2633–2637.
20. Chien, Y.-H. & Dawid, I. B. (1984) *Mol. Cell. Biol.* **4**, 507–513.
21. Richards, R. I., Heguy, A. & Karin, M. (1984) *Cell* **37**, 263–272.
22. Durnam, D. M., Perrin, F., Gannon, F. & Palmiter, R. D. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6511–6515.
23. Lusky, M. & Botchan, M. R. (1984) *Cell* **36**, 391–401.
24. Gluzman, Y. (1981) *Cell* **23**, 175–182.
25. Harrison, T., Graham, F. & Williams, J. (1977) *Virology* **77**, 319–329.
26. Kimura, S., Esparza, J., Benyesh-Melnick, M. & Schaffer, P. A. (1974) *Intervirology* **3**, 162–169.
27. Melton, A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035–7056.
28. Braam, J., Ulmanen, I. & Krug, R. M. (1983) *Cell* **34**, 609–618.
29. Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182.
30. Lusky, M. & Botchan, M. (1981) *Nature (London)* **293**, 79–81.