Long-Term Replication of Sendai Virus Defective Interfering Particle Nucleocapsids in Stable Helper Cell Lines

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An essential prerequisite for generating a stable helper cell line, which constitutively expresses functional Sendai virus RNA-dependent RNA polymerase, is the expression of all three Sendai virus nucleocapsid (NC) proteins, NP, P, and L, simultaneously. Generating a stable helper cell line was accomplished by cotransfecting cell line 293 with all three corresponding viral genes under the control of cytomegalovirus promoter-enhancer elements. Cotransfection with a dominant selectable marker enabled selection for stably transfected cells. The levels of the expressed P and NP proteins reached up to 1/10th and 1/20th of the protein levels in Sendai virus-infected cells, respectively. The Sendai virus polymerase activity of the coexpressed proteins was demonstrated by an in vivo polymerase assay. The cell clone H29 gave the strongest signal and produced DI genomes continuously for at least 3 months. This result demonstrates that it is possible to stably express adequate levels of all three viral NC proteins to form Sendai virus polymerase activity, thereby performing the replication and encapsidation of viral RNA, essential prerequisites for a helper cell line to be competent in producing recombinant viruses.

Sendai virus, the prototype of the parainfluenza viruses, is an enveloped negative-strand RNA virus with an unsegmented genome coding for six genes. This group of viruses was recently designated Mononegavirales (13). The transcription and replication of the viral RNA take place in viral ribonucleoprotein complexes in the cytoplasm of infected cells (25). These complexes are called nucleocapsids (NCs) and consist of the viral genomic RNA tightly associated with several thousand molecules of NP protein (60 kDa) to form an RNase-resistant helical structure (25). The associated proteins P (79 kDa) and L (253 kDa) are present at lower amounts in the NC. These two proteins are distributed in clusters throughout the NC core (37, 38). All three of these NC proteins are necessary to retain polymerase activity by using viral RNA as a template. This fact was shown by reconstitution of stripped NCs (18) and by synthesis of viral defective interfering (DI) particle RNA (7).

Over the past few years, several methods aimed at generating cDNA-derived recombinant viruses have been established. In contrast to positive-strand RNA viruses, the transfected recombinant RNA genome cannot be translated directly, since monocistronic viral mRNAs first have to be transcribed from the recombinant viral RNA genome (25). Transcription can be achieved only by the authentic viral polymerase. More recently, two systems, each using a helper virus, have been used to provide the required NC proteins for transcribing and replicating viral RNA sequences. One of these systems used the homologous wild-type (wt) virus (5a, 9, 28, 34), and the other took advantage of the cytoplasmic expression of phage T7 polymerase to express genes on T7 promoter-driven plasmids (3, 6, 36). In both systems, it is possible to obtain genome-like RNA transcripts and a Sendai virus polymerase-dependent expression of the genes encoded by the genome-like RNA. With these systems, several experiments to characterize cis-acting viral elements and functions of nonstructural viral proteins were performed (4, 5, 8, 35, 36). Nevertheless, so far these systems have not succeeded in rescuing cDNA-derived viruses. The lack of success might be attributed to the short period of time for expression of helper function, since the helper viruses kill the cells within a few days. Another reason might be the problem of separating the generated recombinant virus from the helper virus, since the helper virus is always predominant (exception, influenza virus) (4).

We aimed to provide Sendai virus polymerase in an cellular system without the aid of any helper virus. The experimental system involved stably transfected cells and plasmid-based expression vectors for all three NC proteins. We elected to use the pSC-based expression system (24) and cell line 293 (19). High levels of expression were achieved because the adenovirus-transformed 293 cells are able to transactivate the human cytomegalovirus promoter-enhancer region (16, 17), which is present on the pSC expression vector. The expression plasmids for NP, P, and L protein (pSC-NP, pSC-P, and pSC-L, respectively) were constructed by standard techniques (39) with cloned viral genes of Sendai virus (Fushimi strain, ATCC VR105) (15, 32, 33).

All three expression plasmids coding for NP, P, and L proteins were cotransfected into 293 cells along with pSV2neo which provides a selectable marker (Neo*). In order to obtain appropriate molar quantities of the NC proteins, the amounts of the plasmids coding for NP, P, and L proteins were 5:5:2, respectively, according to the results of transient expression studies (23). More than 200 G418-resistant individual clones were isolated. Identification of cells expressing viral proteins was performed by immunoblotting by the method described in the legend to Fig. 1. Half of the isolated cell clones expressed detectable amounts of P protein, and nearly 50% expressed NP protein simultaneously (data not shown). This result was surprising, since the frequency of simultaneous protein expression of two unrelated transfected genes is reported to be approximately 10% (29). The higher frequency (50%) we found for cell clones stably expressing several transfected genes along with the G418 resistance was assumed to be attributed to our optimized transfection conditions or to...
specific characteristics of the 293 cells. For transfection, we modified the lipofection protocol (11, 12) by using, unconvensionally, Dulbecco’s modified Eagle medium (DMEM) with fetal calf serum (1). This modification probably improves the expression level by increasing cell viability during transfection. The other point is that in different cell lines, transfected DNA is retained at very different frequencies (21, 29), and we chose 293 cells, since they have been proven to express transfected DNA for a long period.

The expression levels of the proteins NP and P vary widely between individual clones. Cell clones with high levels of simultaneous expression of NP and P protein (H29, H30, H31, and H32) are shown (Fig. 1). The maximal levels of P and NP protein were 6 and 8 ng/μg of total cellular protein, respectively (Table 1), as demonstrated by densitometric scanning. Comparison with the values for viral proteins in Sendai virus-infected cells (P protein, 39 ng/μg; NP protein, 118 ng/μg) reveals that in the transfected cell clones, up to 14.6 and 7% of P and NP protein, respectively, is present (Table 1). The level of L protein was below the detection limit of 0.4 ng/μg of total cellular protein with a specific monoclonal antibody (10) or polyclonal antiserum. To determine whether any of these clones express mRNA for the L protein, we screened the genomic DNAs of the clones by PCR for L-specific DNA and then looked for L-specific mRNA by reverse transcription PCR (RT-PCR) of oligo(dT)-selected RNA. In 8 of 20 clones that express NP and P protein, L-specific mRNA could be detected. In some of these clones, L-specific mRNA was expected to be of full length and therefore should be translatable into intact L protein.

Confirmation of the presence of L protein was performed by an in vivo polymerase assay similar to that described by J. Curran et al. (7). The functionality of the Sendai virus polymerase analyzed in that assay depends on the presence of all three NC proteins, NP, P, and L, and the viral NC as the template (7). The NC proteins have to be expressed at specific levels relative to each other as demonstrated in an in vitro polymerase assay (23). As the template for the polymerase, the DI particle DI-H4 was used, a copy-back DI particle, containing a genome of 1,410 nucleotides (3, 4, 27). This assay relies on the dependence of DI particles for helper-supplemented polymerase to replicate. However, since DI particle preparations always contain wt virus, these nondefective genomes were inactivated by UV light. The resulting DI-H4<sup>wt</sup> was used to infect cells, and at 3 days postinfection, RNA was isolated to detect DI particle RNA by RT-PCR. Taking into consideration the copy-back organization of the DI genome, we used a pair of primers, both in the genomic sense located exactly at the 3’ terminus and near the 5’ terminus of the DI genome. RT-PCR amplification resulted in a DNA fragment of 1,147 bp.

The assay system is validated by showing that DI-H4<sup>wt</sup> is not able to replicate in cells without helper function by infecting 293 cells (Fig. 2A, 293 lane –) and is able to replicate in 293 cells at coinfection with wt virus (Fig. 2A, 293 lane wt). As a positive control for expressing plasmid-derived viral polymerase, the transient expression system based on infection

<table>
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<tr>
<th>Cell</th>
<th>Protein level (ng/μg of total protein)</th>
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<tbody>
<tr>
<td></td>
<td>NP</td>
</tr>
<tr>
<td>H29</td>
<td>5.7 (4.8)</td>
</tr>
<tr>
<td>H30</td>
<td>4.3 (3.6)</td>
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<tr>
<td>H31</td>
<td>8.3 (7.0)</td>
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<tr>
<td>H32</td>
<td>6.9 (5.8)</td>
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<tr>
<td>Sendai virus-infected CV1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>118</td>
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<sup>a</sup> Quantitation was done on the basis of the immunoblot shown in Fig. 1. As a standard for quantitation, purified Sendai virus particles were used. The quantitation of the NP, P, and L protein is based on the corresponding protein amounts in virus particles as determined by Lamb and Choppin (26).

<sup>b</sup> Because L protein could not be detected in the cell clones, the detection limit (0.4 ng/μg of total protein) is given in the table.

<sup>c</sup> The percentage of expressed protein in H29 relative to viral proteins in infected cells is shown in parentheses. For evaluating the expression in the stably transfected cell clones, the levels of viral proteins in infected CV1 cells were set at 100%; the percentage of viral proteins in transfected cells has been calculated relative to this value.

<sup>d</sup> The immunoblot for Sendai virus-infected CV1 cells is not shown in this article.
A recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) and subsequent transfection of plasmids coding for the three Sendai virus proteins (NP, P, and L) was used (14). Transfection of all three plasmids resulted in a strong signal (Fig. 2A, 293 lane transient NP,P,L), demonstrating expression of Sendai virus polymerase activity. Omitting the plasmid for L protein almost completely abolished the RNA synthesis (Fig. 2A, 293 lane transient NP,P). The remaining very faint signal of RNA synthesis seems to depend on polymerase activity associated with the input NCs.

The in vivo polymerase assay was used to screen for polymerase activity in all 20 cell clones that stably expressed immunologically detectable amounts of NP and P proteins, including the 8 cell clones with detectable L mRNA. Within these cell clones, three (H29, H30, and H32) were found to be able to synthesize DI particle RNA at various degrees of efficiency (Fig. 2A). We conclude, therefore, that intact L protein is expressed in these clones and that in association with NP and P protein it generates the functional polymerase complex.

The length of the synthesized DI specific RNA was determined by analysis of total cellular RNA after DI-H4UV infection by Northern (RNA) blotting (Fig. 2B). The size of DI-specific RNA in H29 cells is the same as that of authentic DI particle RNA (lane 293 wt). Therefore, the functionality of the expressed polymerase to synthesize full-length DI genomes was confirmed.

To discriminate in H29 cells between simple RNA synthesis and authentic viral genome replication, represented by synthesis of full-length genome transcripts encapsidated as functional NCs, we first analyzed the abilities of the transfected cell lines to form nucleocapsid-like particles, as demonstrated by Buchholz et al. (2) and Spehner et al. (42). Then we addressed the question whether these newly synthesized NCs were suitable to serve as templates for further rounds of replication. Sendai virus nucleocapsid-like particles were purified by banding in a linear cesium chloride density gradient and analyzed by immunoblotting (2). Detection of NP and P proteins in the NC fraction of DI-H4UV-infected H29 cells confirmed the formation of nucleocapsid-like particles in these cells (Fig. 3, lane H29). As controls, cell clones H3 and H55 which express only P or NP protein, respectively, were used. With H3 cells, only a very faint signal could be detected, probably representing the input NCs, demonstrating that P protein alone is insufficient to form new nucleocapsid-like structures (Fig. 3, lane H3). In contrast, H55 cells produce nucleocapsid-like particles showing in addition to current knowledge (2, 42) that even relative low expression levels, as obtained here in stably transfected
cells, are sufficient to induce spontaneous formation of nucleocapsid-like particles (Fig. 3, lane H55).

It is shown by Buchholz et al. (2), that nucleocapsid-like particles contain unspecific nonviral RNA. To prove whether the nucleocapsid-like particles found in cell line H29 infected with DI-H4uv contain correctly encapsidated viral genomic RNA, we infected H29 and H3 cells with DI-H4uv and propagated them for up to 3 months. From these continuous cultures, we purified NCs 3 days, 30 days (4 passages), and 90 days (12 passages) postinfection and isolated RNA as described above. The RNA was analyzed by Northern blotting with a DI-H4 specific probe (Fig. 4). DI particle RNA could be detected in cell clone H29 at each point of analysis, whereas in the control H3 cells (expressing only P protein), no signal was obtained. This result demonstrates that NCs in DI-H4uv-infected H29 cells are present over a long time period. The intensities of the signals on the Northern blot are not comparable, because the blots were performed consecutively. Moreover, changes in the culture of the cells would have to be considered. Splitting (1:20) of the cells was performed every week, and since nonreplicated NCs have a life span in cells of only up to 4 days (31), the persisting NCs are undoubtedly the replication products of multiple replication rounds. Therefore, we were able to demonstrate in H29 cells the synthesis of NCs which were fully functional in terms of the ability to provide polymerase activity and to serve as the template for further rounds of replication. This synthesis of replicable NCs in H29 cells is in addition to the ability to synthesize and encapsidate viral RNA, an important prerequisite for H29 cells to serve as a helper cell line in generating recombinant viruses.

Until now, no experimental system has been available to rescue plasmid-derived recombinant viruses. To generate plasmid-derived viral RNA analogs, there are two systems available, but both are triggered by helper viruses. The system that uses the homologous wt virus as the helper virus provides no possibility for separating the plasmid-derived viral particles from the helper virus by any physical or biological means (5, 9, 34). The other system that uses the recombinant vaccinia virus vTF7-3 reduces the life span of the infected helper cells because of virus-mediated cytotoxicity which possibly hinders the generation of recombinant viruses, especially slowly growing viral mutants. By using the vaccinia virus as the helper, an additional hindrance for generating recombinant viruses might exist, since the ineffectiveness of helper function provided by the wt Sendai virus in the presence of vaccinia virus infection has been observed (3). Possibly, a vaccinia virus-induced factor interferes with the replication of the Sendai virus.

The problems mentioned above could be overcome by using a stable recombinant cell line that provides Sendai virus polymerase functions, such as our H29 cell line. We have demonstrated that these cells perform the replication and encapsidation of viral RNA. Particularly, the capacity to replicate viral NCs for many weeks makes these cells advisable as tool for generating recombinant *Mononegavirales*, even for slowly growing mutants.

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