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Infectious RNA transcribed *in vitro* from a cDNA copy of the human coronavirus genome cloned in vaccinia virus

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The coronavirus genome is a positive-strand RNA of extraordinary size and complexity. It is composed of approximately 30000 nucleotides and it is the largest known autonomously replicating RNA. It is also remarkable in that more than two-thirds of the genome is devoted to encoding proteins involved in the replication and transcription of viral RNA. Here, a reverse-genetic system is described for the generation of recombinant coronaviruses. This system is based upon the *in vitro* transcription of infectious RNA from a cDNA copy of the human coronavirus 229E genome that has been cloned and propagated in vaccinia virus. This system is expected to provide new insights into the molecular biology and pathogenesis of coronaviruses and to serve as a paradigm for the genetic analysis of large RNA virus genomes. It also provides a starting point for the development of a new class of eukaryotic, multi-gene RNA vectors that are able to express several proteins simultaneously.

Introduction

Coronaviruses are enveloped, vertebrate viruses that are associated mainly with respiratory and enteric diseases. The human coronaviruses are responsible for 10–20% of all common colds (McIntosh, 1996). The virus genome is a positive-strand RNA of approximately 30 kb that encodes a minimal set of four structural proteins and a large array of non-structural proteins involved in replication and transcription (Lai & Cavanagh, 1997; Siddell & Snijder, 1998). These so-called replicase proteins are encoded in two overlapping open reading frames (ORFs) that extend about 20 kb from the 5′ end of the genome. It is a hallmark of coronaviruses that extensive co- and post-translational proteolytic processing is required to produce the proteins needed to assemble a functional replication–transcription complex (Ziebuhr *et al.*, 2000). It is also noteworthy that the generation of coronavirus sub-genomic mRNAs involves an unusual process of discontinuous transcription (Spaan *et al.*, 1983), most probably during the synthesis of subgenomic, negative-strand templates (Sawicki & Sawicki, 1998). Discontinuous transcription is a highly regulated process and is, at least in part, dependent upon base-pairing between *cis*-acting elements, the so-called transcription-associated sequences, located at the 5′ end of the genome and at various 3′-proximal sites (van Marle *et al.*, 1999).

Until recently, the study of coronavirus genetics was essentially restricted to the analysis of temperature-sensitive (*ts*) mutants (Lai & Cavanagh, 1997; Stalcup *et al.*, 1998), the analysis of defective RNA templates that depend upon replicase proteins provided by a helper virus (Repass & Makino, 1998; Izeta *et al.*, 1999; Williams *et al.*, 1999) and the analysis of chimeric viruses generated by targetted recombination (Fischer *et al.*, 1997; Hsue & Masters, 1999; Kuo *et al.*, 2000). This was because the large size of the coronavirus genome and the instability of some coronavirus cDNAs in bacteria effectively precluded the use of cloning procedures that have been used to generate infectious RNA from cDNA copies of other positive-strand RNA virus genomes (Ruggli & Rice, 1999). Recently, however, two different approaches have been developed that appear to overcome these problems. Firstly, Almazán *et al.* (2000) have reported that the cloning of full-length, transmissible gastroenteritis virus (TGEV) cDNA in a bacterial artificial chromosome, combined with nuclear expression of infectious RNA, can be used to produce recombinant virus. Secondly, Yount *et al.* (2000) have described a system to assemble a full-length cDNA construct of the

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TGEV genome by using adjoining cDNA subclones that have unique, flanking, interconnecting junctions. Transcripts derived from the TGEV cDNA assembled in this way can be used to derive infectious recombinant virus.

Despite the remarkable achievements of Almazán *et al.* (2000) and Yount *et al.* (2000), we have been unable to construct a stable, full-length cDNA copy of the genome of either the human coronavirus strain 229E (HCoV) or murine hepatitis virus (MHV) using plasmids, bacterial artificial chromosomes, bacteriophage vectors or an *in vitro* approach based upon long-range RT–PCR (Thiel *et al.*, 1997; Herold *et al.*, 1998). We, therefore, decided to pursue an alternative strategy based upon the optimization of *in vitro* DNA ligation, the use of vaccinia virus as a eukaryotic cloning vector and the cytoplasmic expression of transfected RNA that has been transcribed *in vitro*. We reasoned that this approach would have several advantages. Firstly, poxvirus vectors are eminently suitable for the cloning of large cDNAs. It has been shown that they have the capacity to accept at least 26 kbp of foreign DNA (Smith & Moss, 1983) and recombinant vaccinia genomes of this size are stable, infectious and replicate in tissue culture to the same titre as non-recombinant virus. Secondly, vaccinia virus vectors have been developed that are designed for the insertion of foreign DNA by *in vitro* ligation (Merchlinsky & Moss, 1992). This obviates the need for plasmid intermediates carrying the entire cDNA insert. Thirdly, using this approach, recombinant virus is recovered from an infectious RNA that is introduced and replicates in the cytoplasm of the transfected cell. Thus, there are no concerns regarding RNA modification, processing and export from or degradation within the nucleus.

In this study, we show that human coronavirus cDNA fragments of more than 27 kbp can be stably cloned and propagated in vaccinia virus. Moreover, a recombinant vaccinia virus clone, containing a full-length HCoV cDNA, enabled us to produce infectious *in vitro* RNA transcripts and to rescue recombinant human coronavirus.

Methods

■ **Cells and virus and RNA transfection.** Human lung fibroblast (MRC-5), monkey kidney fibroblast (CV-1) and human cervix epithelial (HeLa-S3) cells were purchased from the European Collection of Cell Cultures and maintained in minimum essential medium (MEM) supplemented with HEPES (25 mM), foetal bovine serum (5–10%) and antibiotics. HCoV 229E, vaccinia virus strain v*Not*I/tk (Merchlinsky & Moss, 1992) and vaccinia virus recombinants were propagated, titrated and purified by using standard procedures (Raabe *et al.*, 1990; Mackett *et al.*, 1985). Fowlpox virus strain HP1.441 (Mayr & Malicki, 1966) was propagated in chicken embryo fibroblast cells that were maintained in MEM supplemented with 7% foetal bovine serum.

■ **Transfection.** CV-1 cells were grown to 80% confluence and transfected for 2 h at 37 °C with 1–5 μg *in vitro*-ligated DNA and 10 μl lipofectin in OPTIMEM 1, according to the supplier's instructions (Life Technologies). MRC-5 cells were grown to 80% confluence and transfected for 30 min at 37 °C with 1 μg *in vitro*-transcribed RNA and 10 μl lipofectin in OPTIMEM 1, according to the supplier's instructions.

■ **Preparation of poly(A)-containing RNA and preparative RT–PCR.** Poly(A)-containing RNA was isolated from coronavirus-infected MRC-5 cells by using oligo$(dT)_{25}$ Dynabeads as described by Thiel *et al.* (1997). RT–PCR was also done as described by Thiel *et al.* (1997) with Superscript II reverse transcriptase (Life Technologies) and native *Pfu* thermostable DNA polymerase (Stratagene). To produce the DNA fragment PCR-BF, three oligonucleotide primers were used: 5′ CTACTCACGATATCGTAC 3′ (nt 7840–7858, reverse transcription), 5′ AGTTGGTGTTATTGCTGATAAGGAC 3′ (nt 5176–5200, PCR) and 5′ GACATAGGCCGGCCCTGTTGGTTGCACATTTGTTTTG-GT 3′ (nt 6968–7006, PCR). The PCR-BF fragment comprises 1830 bp representing positions 5176–7006 in the HCoV genome. The PCR-BF fragment is flanked by a natural 5′ *Bgl*II site (nt 5203–5208) and a 3′ *Fse*I site present in the PCR primer (nt 6993–7000). In order to identify diagnostic mutations in the recombinant HCoV genome, three oligonucleotides primers were used: 5′ CTACTCACGATATCGTAC 3′ (nt 7840–7858, reverse transcription), 5′ CAACTTGATGAAAAGG-CAC 3′ (nt 6032–6050, PCR) and 5′ AACCTCTTTGCAAGAATA-CTTGCT 3′ (nt 7094–7117, PCR and sequencing).

■ **Gel electrophoresis.** RNAs were fractionated by electrophoresis in 0·8% agarose/TBE gels containing 0·1% SDS (TBE is 89 mM Tris–HCl, 89 mM borate, 2 mM EDTA, pH 8·3). Smaller DNA fragments were resolved by electrophoresis in 0·6–1·0% agarose/TBE gels. Larger DNA fragments were resolved by pulsed-field gel electrophoresis in the CHEF-DR III system (Bio-Rad) using 1% agarose/0·5 × TBE gels at 14 °C with a switch time of 3–30 s, a run time of 18 h and 6 V/cm at an angle of 120°. RNA and large DNA samples were heated to 65 °C for 10 min prior to electrophoresis. Gels were stained with ethidium bromide after electrophoresis.

■ **Northern and Southern blots, PCR and sequence analysis.** Poly(A)$^+$ RNA from HCoV-infected MRC-5 cells was electrophoresed on 2·2 M formaldehyde–1% agarose gels. The gels were dried and hybridized to 5′-end ^{32}P-labelled oligonucleotides as described by Meinkoth & Wahl (1984). The oligonucleotide 5′ AGAAACTTCA-TCACGCACTGG 3′ (nt 26802–26822) was used to detect HCoV genomic and subgenomic RNAs. The oligonucleotide 5′ ACATAC-GCTGGGCCTGTT 3′ (nt 6988–7005) was used to detect the parental HCoV 229E genomic RNA. The oligonucleotide 5′ ACATAGGCC-GGCCCTGTT 3′ (nt 6988–7005) was used to detect the recombinant HCoV-inf-1 genomic RNA.

CV-1 cells (1×10^5) were infected with parental or recombinant vaccinia virus and incubated until cytopathic effects were evident. The cells were harvested and incubated for 2 h at 50 °C with 200 μl proteinase K buffer (0·1 mg/ml proteinase K in 100 mM Tris–HCl, pH 7·5, 5 mM EDTA, 0·2% SDS, 200 mM NaCl). The digest was then deproteinized with phenol–chloroform extraction and precipitated with ethanol. The DNA was digested overnight at 37 °C with *Hin*dIII and the resulting fragments were electrophoresed and transferred to nylon membranes as described by Ausubel *et al.* (1987). The Multiprime DNA-labelling system was used as recommended by the supplier (Amersham) to produce two ^{32}P-labelled probes from DNA templates. The templates were a 19 kb RT–PCR product corresponding to nt 1048–20582 of the HCoV genome and a PCR product corresponding to nt 23850–26822 (V. Thiel, unpublished). The probes were mixed and hybridized to the immobilized DNA fragments by standard methods (Ausubel *et al.*, 1987).

In addition to Southern blot analysis, recombinant vaccinia virus DNAs were screened by PCR analysis. To do this, DNA from infected

CV-1 cells was prepared as described above and used as a template for a standard PCR using thermostable *Taq* DNA polymerase and the oligonucleotides 5′ CCAGGCTGGAGTCTGCAG 3′ (nt 22491–22508) and 5′ GACAACTAGGTCTGGAAC 3′ (nt 23723–23740).

Sequencing of plasmid constructs, RT–PCR products and the recombinant vaccinia virus cDNA insert was done by standard cycle-sequencing methods using the BigDye Terminator kit (Applied Biosystems). The analysis of sequencing products was done by capillary electrophoresis using an ABI 310 PRISM Genetic Analyser. Computer-assisted analysis of sequence data was facilitated by the LASERGENE bio-computing software (DNASTAR).

■ **Plasmid construction.** Plasmids were constructed from a library of HCoV 229E cDNA clones and RT–PCR products by standard procedures (Ausubel *et al.*, 1987). The precise details of these procedures are available from the authors upon request. The plasmid pEB is based on pBluescript II KS+ and contains sequences corresponding to nt 1–5207 of the HCoV 229E genome, preceded by an additional G nucleotide, the sequence for the bacteriophage T7 RNA polymerase promoter and *Bsp*120I and *Eag*I restriction sites. The plasmids pME and pFE are based on pBR322. pFE contains sequences corresponding to nt 6993–20569 of the HCoV genome followed by the green fluorescent protein gene, HCoV 229E sequences from nt 26279 to 27277, a synthetic poly(A) tail of approximately 40 nt and *Cla*I, *Bsp*120I and *Eag*I restriction sites. The nucleotides at positions 6994, 6997 and 7000 of pFE were mutated from their original sequence. These mutations result in a silent *Fse*I site that is useful for both cloning and diagnostic purposes. pME contains sequences corresponding to nt 12677–27277 of the HCoV 229E genome, a synthetic poly(A) tail of approximately 40 nt and restriction sites for *Cla*I, *Bsp*120I and *Eag*I.

■ **Cloning in vaccinia virus.** Plasmid and RT–PCR DNA fragments were purified with QIAEX II resin following separation by agarose gel electrophoresis. Ligation reactions containing vaccinia virus DNA were analysed by pulsed-field agarose gel electrophoresis. Ligation reactions containing *Not*I were incubated for 16 h at 25 °C in the recommended digestion buffer (New England Biolabs) supplemented with 1 mM ATP. Subsequently, the T4 DNA ligase was heat-inactivated and the incubation was continued for an additional hour at 37 °C with additional *Not*I enzyme.

Construction of the full-length HCoV cDNA was carried out in two steps (Fig. 1). Firstly, cDNA insert fragments EB and FE were derived from the plasmids pEB and pFE by digestion with *Eag*I/*Bgl*II and *Fse*I/*Eag*I, respectively, and treated with alkaline phosphatase. Fragment EB was then ligated with PCR-BF that had been digested with *Bgl*II. The resulting 7 kbp ligation product was digested with *Fse*I, purified by agarose gel electrophoresis and ligated with fragment FE to produce a 22·5 kbp cDNA. This cDNA fragment was ligated without further purification to v*Not*I/tk vaccinia virus DNA in the presence of *Not*I enzyme. The ligation products were then transfected with lipofectin into CV-1 cells that had been infected 1 h previously with fowlpox virus at an m.o.i. of 5. Two h later, the cells were harvested and replated into 96-well tissue culture dishes with a 4-fold excess of non-transfected, non-infected CV-1 cells. Recombinant vaccinia viruses were isolated from 96 wells that developed cytopathic effect within 2 weeks, plaque purified and analysed by PCR and Southern blots. The sequence of the 22·5 kbp cDNA insert was determined from one such recombinant vaccinia virus, vHCoV-vec-1 (V. Thiel, unpublished results). In a second phase, a cDNA fragment of 22·5 kbp was obtained by digestion of purified vHCoV-vec-1 genomic DNA with *Bsp*120I, dephosphorylated by alkaline phosphatase and purified by gel electrophoresis. After digestion with *Mlu*I and heat inactivation of the *Mlu*I enzyme, the resulting fragments of 12·7 and 9·6 kbp were ligated to a cDNA fragment of pME that was obtained by digestion with *Mlu*I/*Eag*I, treatment with alkaline phosphatase and purification by gel electrophoresis. This produced, amongst other products, a 27·3 kbp cDNA fragment containing the full-length cDNA of the HCoV 229E genome. The ligation products were then ligated to v*Not*I/tk vaccinia virus DNA without further purification in the presence of *Not*I enzyme. By using the rescue procedure described above, a recombinant vaccinia virus, vHCoV-inf-1, was recovered, plaque purified and characterized by PCR and Southern blot analysis. The sequence of the 27·3 kbp cDNA insert of vaccinia virus vHCoV-inf-1 was determined and has been deposited in the GenBank database.

■ ***In vitro* transcription.** DNA was prepared from purified recombinant vaccinia virus vHCoV-inf-1 by proteinase K/phenol treatment (Ausubel *et al.*, 1987) and ethanol precipitation. The genomic DNA was cleaved with *Cla*I enzyme and deproteinized by phenol extraction and ethanol precipitation. *In vitro* transcription of capped RNA was done by using a RiboMAX kit. The reaction was based upon the conditions recommended by the supplier (Promega) and contained 5–10 μg *Cla*I-cleaved vHCoV-inf-1 DNA and m7G(5′)ppp(5′)G RNA (cap analogue) at a ratio of 1:1 with GTP. The *in vitro* transcription reaction was incubated at 25 °C for 2·5 h and was followed by DNase I treatment and RNA precipitation.

Results

The overall strategy of this study is illustrated in Fig. 2. Briefly, cDNAs representing the entire HCoV genomic RNA were assembled by *in vitro* ligation. The cDNA was then ligated, again *in vitro*, to the left and right arms of a vaccinia virus genomic DNA that had been cleaved at a unique *Not*I site. The recombinant vaccinia virus DNA was transfected into CV-1 cells and recombinant vaccinia viruses were rescued by co-infection with fowlpox virus. After the isolation of a recombinant vaccinia virus containing a full-length HCoV genomic cDNA, the recombinant vaccinia virus DNA was purified and used as a template for the *in vitro* transcription of recombinant HCoV genomic RNA. This RNA was transfected into MRC-5 cells and the cultures were monitored for the recovery of recombinant coronavirus.

Cloning and propagation of HCoV cDNA in vaccinia virus

One of the major goals in this study was to establish a protocol that enabled us to introduce large coronavirus cDNAs into the vaccinia virus genome. First, we produced a set of plasmid clones, pEB, pFE and pME, that contained extensive segments of the HCoV genome. These plasmids were assembled from a collection of well-characterized cDNA clones (Herold *et al.*, 1993; Raabe *et al.*, 1990; Raabe & Siddell, 1989*a*, *b*; Myint *et al.*, 1990; S. Siddell, unpublished). Next, we constructed and cloned in vaccinia virus a 22·5 kbp HCoV cDNA insert, HCoV-vec-1. The construction and cloning of the cDNA will be described in detail elsewhere (V. Thiel, unpublished results) but is described here, briefly, because it represents an important intermediate in the derivation of the

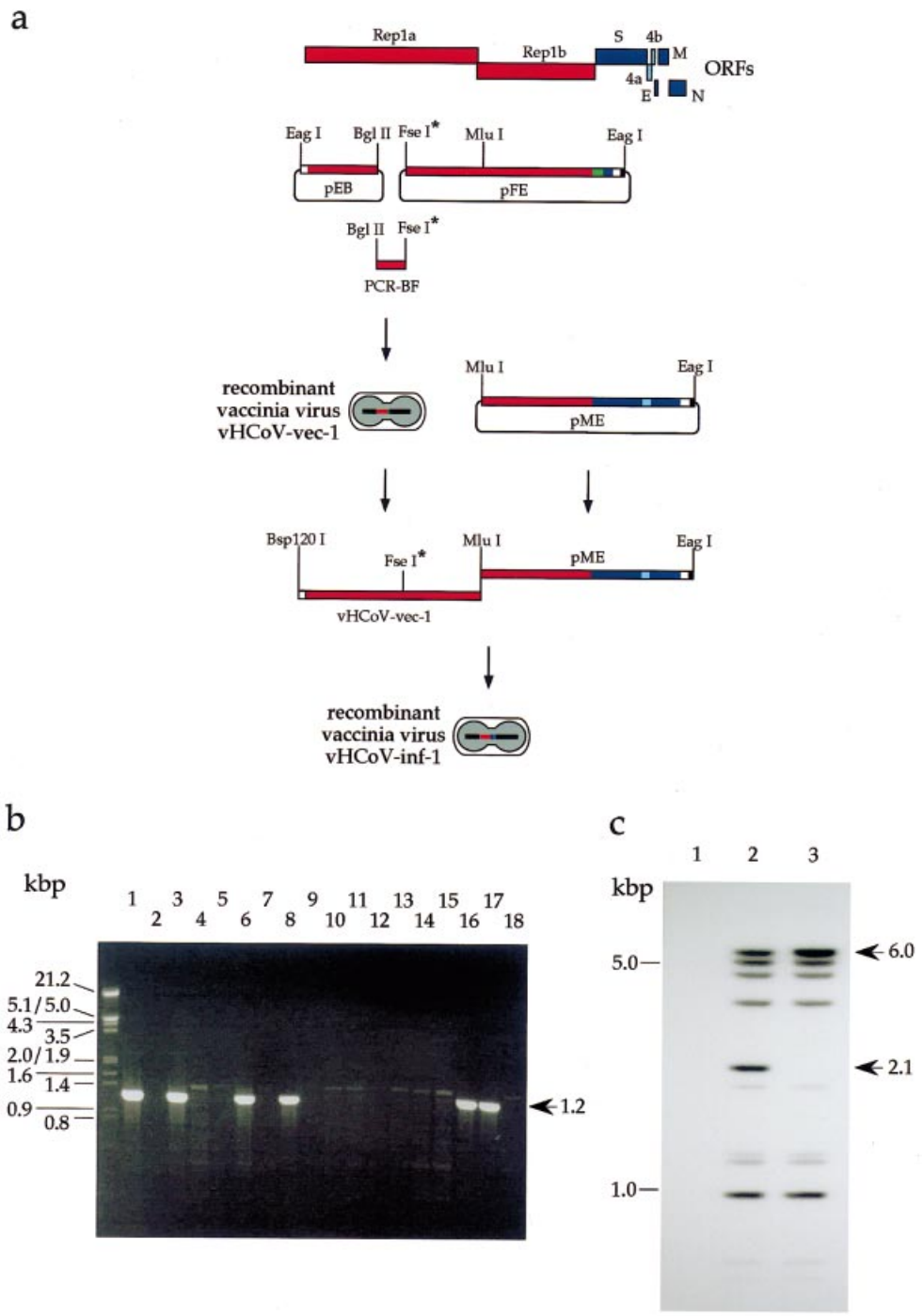

Fig. 1. Cloning of full-length HCoV cDNA in the vaccinia virus genome. (*a*) The two-phase strategy used to generate a full-length HCoV cDNA. The structural relationship of the HCoV 229E ORFs, the plasmid DNA and vaccinia virus HCoV-vec-1 inserts used to assemble the full-length HCoV cDNA is shown. The relevant restriction sites are indicated. ORFs encoding virus replicase proteins are coloured red. Virus structural protein genes are coloured dark blue and virus non-structural protein genes are coloured light blue. Fragment pFE also contains a region encoding green fluorescent protein, which is coloured green. (*b*) PCR analysis of 16 random recombinant vaccinia virus genomic DNAs. The templates for the PCRs were: lane 1, pME plasmid DNA; lane 2, water; lanes 3–18, DNA from rescued vaccinia virus plaques. *Hin*dIII/*Eco*RI-digested lambda DNA is shown as a size marker. The indicated PCR product of 1249 bp (lanes 3, 6, 8, 16 and 17) is amplified from a region within the surface protein gene (nt 22491–23740) that is present only in recombinant vaccinia viruses containing the full-length HCoV-229E cDNA insert. (*c*) Southern blot analysis of two selected vaccinia virus vHCoV-inf clones containing full-length cDNA inserts in different orientations. DNA from CV-1 cells infected with v*Not*I/tk (lane 1), recombinant vHCoV-inf-1 (lane 2) or recombinant vHCoV-inf-2 (lane 3) was digested with *Hin*dIII and analysed by Southern blotting using two random-primed

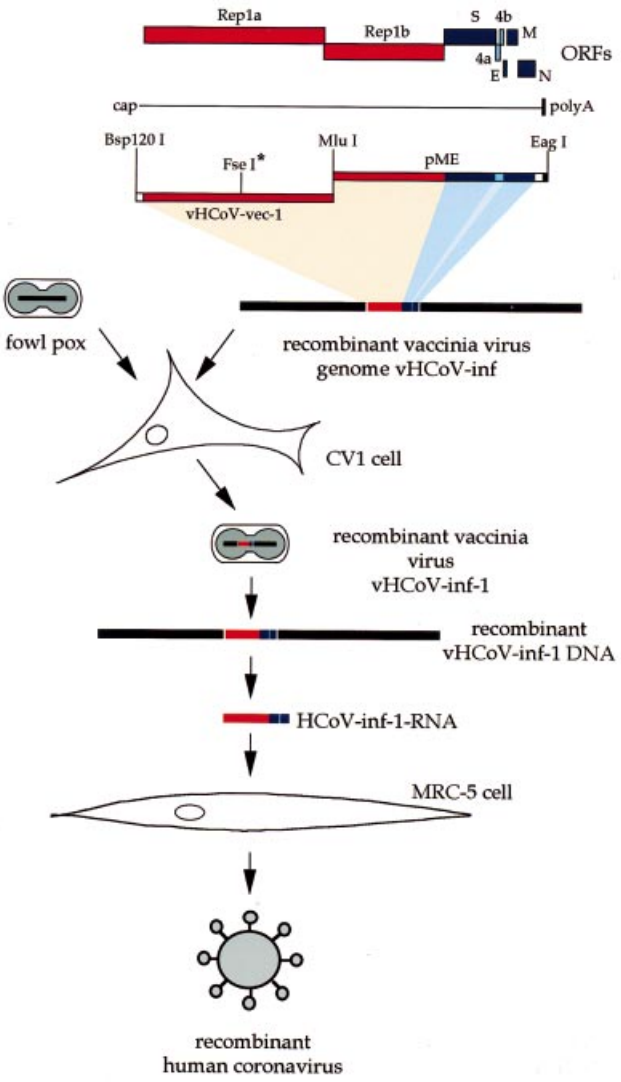

Fig. 2. Strategy for the production of infectious HCoV 229E RNA and the recovery of recombinant coronavirus. The structural relationship of the HCoV 229E ORFs, HCoV 229E genomic RNA and the HCoV-inf-1 cDNA is shown. Two cDNA fragments, derived from vHCoV-vec-1 and pME, are assembled by *in vitro* ligation using an *Mlu*I restriction site. Subsequent ligation of the resulting cDNA with *Not*I-cleaved v*Not*I/tk vaccinia virus DNA produces the recombinant vaccinia virus vHCoV-inf DNA. This DNA is then transfected into CV-1 cells and the recombinant vaccinia virus vHCoV-inf-1 is recovered by using fowlpox helper virus. RNA transcripts are produced *in vitro* by using genomic vHCoV-inf-1 DNA and bacteriophage T7 RNA polymerase and transfected into MRC-5 cells. Finally, tissue cultures are monitored for the production of recombinant human coronavirus.

full-length HCoV genomic cDNA (Fig. 1*a*). First, we ligated cDNA fragments prepared from plasmids pEB and pFE together with an RT–PCR cDNA product, PCR-BF. PCR-BF encompasses a region of the HCoV genome (circa nt 5200–7000) that we are unable to maintain as a plasmid cDNA in bacteria. The products of this reaction were then ligated with vaccinia virus v*Not*I/tk vector DNA in the presence of *Not*I enzyme. The *in vitro*-ligated, recombinant vaccinia virus DNA was then used to recover a recombinant vaccinia virus, vHCoV-vec-1, using fowlpox virus as described in Methods. After having confirmed the sequence of the vHCoV-vec-1 cDNA insert, it was used as a source for one of the two DNA fragments needed to construct the full-length HCoV genomic cDNA.

In a second phase, the vHCoV-vec-1 genomic DNA was used to produce a fragment, BM, that essentially encompassed the HCoV 5′ NTR and the replicase ORF 1a. The plasmid pME was used to produce a cDNA fragment that encompasses the remainder of the genome (Fig. 1*a*). These fragments, together with vaccinia virus v*Not*I/tk vector DNA, were ligated *in vitro* in the presence of *Not*I enzyme. The reason for adding *Not*I is that it favours the accumulation of recombinant vaccinia virus genomes, rather than the parental vaccinia virus genome (V. Thiel, unpublished results). The ligation reaction products were then used to recover recombinant vaccinia virus plaques as described in Methods. We obtained more than 50 vaccinia virus plaques and analysed 16 of them by PCR. Fig. 1(*b*) shows that, of these 16 plaques, five contained coronavirus cDNA inserts that included the structural surface protein gene. Southern blot analysis of the genomic DNA of these five recombinant vaccinia viruses indicated that they all contained single-copy, full-length HCoV cDNA (data not shown). Further analysis of two clones, vHCoV-inf-1 and vHCoV-inf-2 (Fig. 1*c*, lanes 2 and 3), confirmed the integrity and orientations of the inserts. Finally, the 27·3 kbp cDNA insert of vHCoV-inf-1 was sequenced and found to be as predicted; this sequence has been deposited in GenBank. Although the insert cDNA of vHCoV-inf-1 exceeds the length of any insert cloned so far into the vaccinia virus genome, this recombinant clone remained stable and infectious and replicated in tissue culture at the same rate and to same titre as standard vaccinia virus (data not shown).

Recovery of a recombinant human coronavirus

To recover a recombinant human coronavirus, we prepared genomic DNA from purified vaccinia virus vHCoV-inf-1, cleaved this DNA with *Cla*I enzyme and transcribed capped RNA *in vitro* using bacteriophage T7 RNA polymerase. As is shown in Fig. 3(*a*), *in vitro* transcription of this DNA at 25 °C gave both a reasonable amount (approximately 50 µg per reaction) and a high proportion of full-length (i.e. 27·3 kb) RNA. We found that higher or lower temperatures were

[32]P-labelled DNA probes representing the HCoV 229E genome between nt 1048–20582 and 23850–26822. The DNA fragments expected from *Hin*dIII-digested, full-length HCoV 229E cDNA (6·0, 5·1, 4·3, 3·2, 1·8, 1·2, 1·2, 1·0, 0·7 and 0·6 kbp; fragments below 0·5 kbp are not detected) are shown. The *Hin*dIII fragments representing the fusion of the 3′ end of the HCoV genomic cDNA and the vaccinia genomic DNA are indicated; the fragments of approximately 2·1 kbp (lane 2) and 6·0 kbp (lane 3) represent the alternative cDNA orientations.

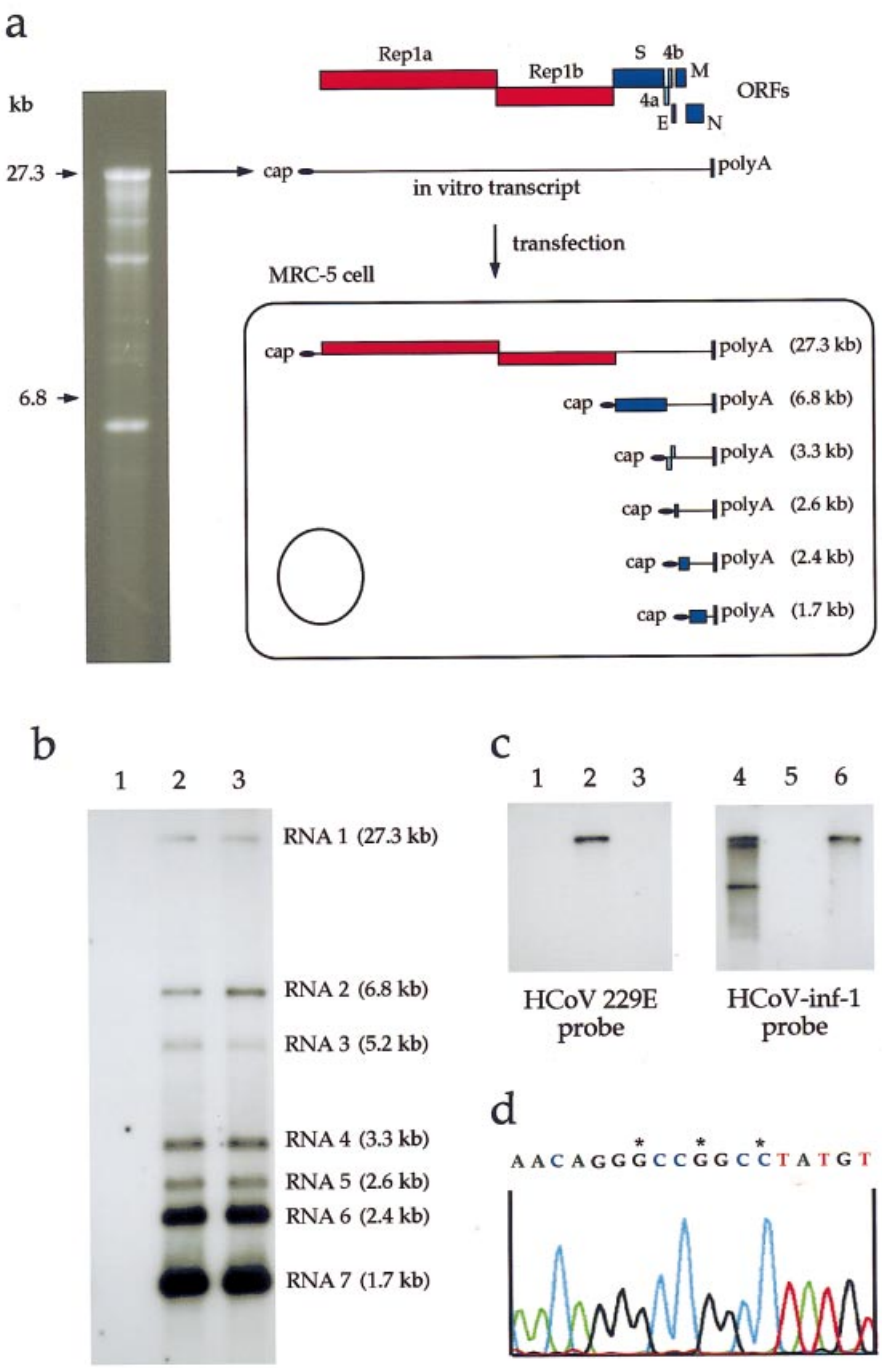

Fig. 3. Recovery of recombinant human coronavirus. (*a*) Ethidium bromide-stained, 1 % agarose gel in which 1 μg capped RNA, transcribed *in vitro* from vHCoV-inf-1 DNA, has been electrophoresed. The full-length (27·3 kb) *in vitro* transcription product is indicated. Also shown is the structural relationship of the HCoV ORFs, the *in vitro*-transcribed HCoV-inf-1 RNA and the predicted genomic and subgenomic mRNAs in HCoV-inf-1 RNA-transfected MRC-5 cells. (*b*) Analysis of poly(A)-containing RNA from parental virus- and recombinant virus-infected cells. Poly(A)-containing RNA was isolated from MRC-5 cells that had been mock-infected (lane 1), infected with parental HCoV 229E virus (lane 2) or infected with recombinant HCoV-inf-1 virus (lane 3). The RNA was analysed by Northern hybridization using a ^{32}P-end-labelled oligonucleotide (5′ AGAAACTTCATCACG-CACTGG 3′) corresponding to nt 26802–26822 within the HCoV nucleocapsid protein gene. The characteristic set of genomic and subgenomic HCoV mRNAs is indicated. (*c*) Northern hybridization of *in vitro*-transcribed HCoV-inf-1 RNA (lanes 1 and 4) and poly(A)-containing RNA from parental HCoV 229E-infected MRC-5 cells (lanes 2 and 5) and HCoV-inf-1-infected MRC-5 cells (lanes 3 and 6). The RNAs were probed with a parental HCoV 229E-specific oligonucleotide, 5′ ACATACGCTGGGCCTGTT 3′ (lanes 1–3), or an HCoV-inf-1-specific oligonucleotide, 5′ ACATAGGCCGGCCCTGTT 3′ (lanes 4–6). The oligonucleotides were ^{32}P-end-labelled. (*d*) Sequence analysis of HCoV-inf-1 genomic RNA in the region encompassing the three silent mutations specific to the recombinant virus genome. The three nucleotide mutations are indicated that represent the diagnostic *Fse*I site.

detrimental to the integrity and/or the yield of the RNA transcripts (data not shown). When this RNA was transfected into MRC-5 cells using lipofection as described in Methods, cytopathic effects characteristic of human coronavirus infection developed throughout the culture after 6–7 days. A virus, which we have designated HCoV-inf-1, was recovered from the tissue culture supernatant, plaque purified and propagated by three or four undiluted passages in MRC-5 cells to produce stocks containing approximately 1×10^7 $TCID_{50}$/ml. The growth kinetics, cytopathic effect and stability of the recovered virus were indistinguishable from those of parental virus (data not shown). These stocks were then used to infect MRC-5 cells at an m.o.i. of 5 and poly(A)-containing RNA was isolated for Northern hybridization analysis. As shown in Fig. 3(*b*), the patterns of genomic and subgenomic RNAs synthesized in HCoV 229E- and HCoV-inf-1-infected cells were identical. Specifically, the characteristic pattern of HCoV genomic and subgenomic mRNAs (RNA1, 27·3 kb; RNA2, 6·8 kb; RNA3, 5·2 kb; RNA4, 3·3 kb; RNA5, 2·6 kb; RNA6, 2·4 kb; RNA7, 1·7 kb) accumulated in both infections with the same kinetics in non-equimolar but constant ratios.

In order to confirm that we had, indeed, recovered a recombinant virus, the viral RNAs isolated from infected cells were probed with two synthetic oligonucleotides that discriminate between the parental sequence (nt ^{6988}AACAGGCCCAGCGUAUGU^{7005}) and the recombinant virus sequence (nt ^{6988}AACAGGGCCGGCCUAUGU^{7005}; i.e. the sequence around the unique *Fse*I site). As shown in Fig. 3(*c*), the oligonucleotides respectively hybridized specifically to the genomes of the parental and the recombinant virus (Fig. 3*c*, lanes 2 and 6). The recombinant virus-specific probe also hybridized to the *in vitro*-transcribed HCoV-inf-1 RNA (Fig. 3*c*, lane 4), whereas the parental virus-specific probe did not (Fig. 3*c*, lane 1). Finally, sequence analysis of an RT–PCR-amplified DNA fragment that encompasses the relevant region of the HCoV-inf-1 genome confirmed the presence of the diagnostic mutations (Fig. 3*d*). These results demonstrate, conclusively, the recovery of a recombinant human coronavirus and they demonstrate that the coronavirus genomic RNA alone is able to initiate a productive infectious cycle.

Discussion

The system we describe here should find wide application in the analysis of the molecular biology and pathogenesis of coronaviruses. We have shown that it is possible to clone a full-length cDNA copy of the human coronavirus genome in the vaccinia virus genome and to produce infectious RNA transcripts from this template. In the long term, this system will improve our ability to control coronavirus infections in humans, livestock and domestic animals.

The basis of the approach taken in this study is the use of vaccinia virus as a cloning vector for large cDNA inserts. In this respect, we believe the vaccinia virus system has a number of advantages. Firstly, we have never observed instability of the cloned insert cDNA in the vaccinia virus system. This is in marked contrast to our experience with bacterial systems, where we regularly encounter instability (for example, the insertion of foreign sequences, the deletion of nucleotides, the rearrangement of inserts and the occurrence of single nucleotide changes) when handling large cDNA clones encompassing specific regions of the coronavirus genome. Furthermore, irrespective of the size of the cDNA insert, we have not seen any differences in the infectivity, growth kinetics or stability of the recombinant vaccinia viruses compared to the parental virus. Secondly, we have shown that large cDNA fragments, assembled by *in vitro* ligation using plasmid DNA, RT–PCR DNA or recombinant vaccinia virus cDNA, can be cloned efficiently into the vaccinia virus genome. By incorporating the *Not*I enzyme in the ligation reactions, we have found that more that 90% of recovered vaccinia viruses are recombinant. This protocol facilitates the isolation of recombinant vaccinia virus clones without the need for selection, it obviates the need for plasmid intermediates carrying full-length insert cDNAs and it represents a very flexible way of introducing defined mutations into large cDNA clones. To improve the system further, we think that it should also be possible to introduce specific mutations rapidly into the cloned viral cDNA by using vaccinia virus-mediated homologous recombination (Moss, 1996) and we hope that it will be possible to develop simplified procedures for the recovery of recombinant coronaviruses from recombinant vaccinia virus genomes. This should then result in a straightforward and universal reverse-genetic approach for RNA viruses with large genomes, such as coronaviruses, closteroviruses (Mawassi *et al.*, 2000) and okaviruses (Cowley *et al.*, 2000).

The reverse-genetic system we have developed will be useful in a number of areas. It will greatly facilitate the analysis of coronavirus RNA replication and transcription. For example, Sawicki and colleagues have recently characterized the phenotypes and genotypes of a collection of temperature-sensitive (*ts*) MHV mutants that are unable to synthesize RNA at the restrictive temperature (S. Sawicki, personal communication). The valuable information obtained by this classical approach can now be complemented by a reverse-genetic approach. Moreover, the system we describe also facilitates, in principle, the analysis of coronavirus replication, independent of the virus life-cycle and without the requirement for receptor-mediated infection. Thus, it can be put to great advantage in the analysis of the virus–host cell interaction in the context of virus replication, transcription, assembly and release.

Secondly, the system we describe will complement existing methods of producing recombinant coronaviruses (Masters, 1999; Almazán *et al.*, 2000; Yount *et al.*, 2000) and significantly advance the analysis of coronavirus pathogenesis. With the systems now available, it should be possible to generate rapidly a large collection of genetically modified coronaviruses; for example, intra- and interspecific chimeric viruses,

viruses with gene inactivations or deletions and viruses with attenuating modifications or supplementary functions. The phenotypes associated with these modifications, at least those that are not lethal, can then be tested in animal models of infection. In particular, this should provide important insights into the relationship between coronavirus infection and the immune response.

Finally, the results we present should also encourage the development of coronavirus vectors for the expression of heterologous proteins. In the long term, we believe that the expression of multiple subgenomic mRNAs in coronavirus-infected cells could form the basis of a vector system that allows the expression of multiple transcriptional units, each encoding a heterologous protein. These features and the autonomy of coronavirus RNA replication could then be exploited in the development of a new class of RNA vaccine vectors (Bredenbeek & Rice, 1992; Mandl *et al.*, 1998).

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