Human respiratory syncytial virus (HRSV) is the most important viral cause of severe respiratory tract disease in infants. Two subgroups (A and B) have been identified, which cocirculate during, or alternate between, yearly epidemics and cause indistinguishable disease. Existing in vitro and in vivo models of HRSV focus almost exclusively on subgroup A viruses. Here, a recombinant (r) subgroup B virus (rHRSV) was generated based on a consensus genome sequence obtained directly from an unpassaged clinical specimen from a hospitalized infant. An additional transcription unit containing the gene encoding enhanced green fluorescent protein (EGFP) was introduced between the phosphoprotein and matrix genes (position 5) of the genome to generate rHRSV-EGFP. The recombinant viruses replicated efficiently in both HEp-2 cells and in well-differentiated normal human bronchial cells grown at air-liquid interface. Intranasal infection of cotton rats (Sigmodon hispidus) resulted in high numbers of EGFP-positive cells in epithelia of the nasal septum and conchae. When administered in a relatively large inoculum volume, the virus also replicated efficiently in bronchiolar epithelial cells and spread extensively in both the upper and lower respiratory tracts. Virus replication was not observed in ciliated epithelial cells of the trachea. This is the first virulent rHRSV strain with the genetic composition of a currently circulating wild-type virus. In vivo tracking of infected cells by means of EGFP fluorescence in the absence of cytopathic changes increases the sensitivity of virus detection in HRSV pathogenesis studies.

**ABSTRACT**

Human respiratory syncytial virus (HRSV) is the most important viral cause of severe respiratory tract disease in infants. Two subgroups (A and B) have been identified, which cocirculate during, or alternate between, yearly epidemics and cause indistinguishable disease. Existing in vitro and in vivo models of HRSV focus almost exclusively on subgroup A viruses. Here, a recombinant (r) subgroup B virus (rHRSV) was generated based on a consensus genome sequence obtained directly from an unpassaged clinical specimen from a hospitalized infant. An additional transcription unit containing the gene encoding enhanced green fluorescent protein (EGFP) was introduced between the phosphoprotein and matrix genes (position 5) of the genome to generate rHRSV-EGFP. The recombinant viruses replicated efficiently in both HEp-2 cells and in well-differentiated normal human bronchial cells grown at air-liquid interface. Intranasal infection of cotton rats (Sigmodon hispidus) resulted in high numbers of EGFP-positive cells in epithelia of the nasal septum and conchae. When administered in a relatively large inoculum volume, the virus also replicated efficiently in bronchiolar epithelial cells and spread extensively in both the upper and lower respiratory tracts. Virus replication was not observed in ciliated epithelial cells of the trachea. This is the first virulent rHRSV strain with the genetic composition of a currently circulating wild-type virus. In vivo tracking of infected cells by means of EGFP fluorescence in the absence of cytopathic changes increases the sensitivity of virus detection in HRSV pathogenesis studies.

**IMPORTANCE**

Virology as a discipline has depended on monitoring cytopathic effects following virus culture in vitro. However, wild-type viruses isolated from patients often do not cause significant changes to infected cells, necessitating blind passage. This can lead to genetic and phenotypic changes and the generation of high-titer, laboratory-adapted viruses with diminished virulence in animal models of disease. To address this, we determined the genome sequence of an unpassaged human respiratory syncytial virus from a sample obtained directly from an infected infant, assembled a molecular clone, and recovered a wild-type recombinant virus. Addition of a gene encoding enhanced green fluorescent protein allowed this wild-type virus to be tracked in primary human cells and living animals in the absence of significant cytopathic effects. Imaging of fluorescent cells proved to be a highly valuable tool for monitoring the spread of virus and may help improve assays for evaluating novel intervention strategies.
which cause indistinguishable disease and cocirculate during, or alternate between, yearly outbreaks (9, 10).

An improved understanding of HRSV pathogenesis would facilitate the development of novel intervention strategies. This requires virulent, well-characterized virus strains of known provenance, which can be evaluated in disease-relevant in vitro and in vivo model systems. Well-differentiated (wd) normal human bronchial epithelial (wd-NHBE) cultures grown at air-liquid interface (ALI) have been identified as a useful in vitro model for HRSV as they contain ciliated cells which are natural HRSV targets (11–13). Such cells provide a valuable bridge from in vitro to in vivo studies. Cotton rats represent a highly susceptible small-animal model for HRSV pathogenesis studies (14). Recently, adult human volunteers were infected with wild-type A strains to assess the effectiveness of HRSV antivirals (15–17). Irrespective of the approach used, it is critical to use naturally circulating cultures to ensure that study outcomes can be correlated with clinical outcomes. A long-standing challenge in virology is that clinical isolates often fail to cause overt cytopathic effect (CPE) in primary cells and in vivo; thus, infected cells must be stained to monitor the infection. This is challenging in vitro and magnified in vivo when low numbers of infected cells are present in tissues, which must be examined using ultrathin sections. These challenges have been addressed by generating recombinant (r) viruses from clinical samples and engineering them to express fluorescent proteins from an additional transcription unit (ATU), permitting novel insights into viral pathogenesis and targeted pathological assessment in appropriate cell lines and animal models (18). To extend these studies, we obtained the genome sequence of HRSV B05, a wild-type subgroup B strain. Assembly of a full-length molecular clone allowed the recovery of recombinant HRSV B05 (rHRSV B05) and insertion of an ATU containing the enhanced green fluorescent protein (EGFP) open reading frame (ORF) at position 5 between the phosphoprotein (P) and matrix (M) genes led to the generation of rHRSV B05EGFP(5). We characterized rHRSV B05 and rHRSV B05EGFP(5) in vitro and show that it is virulent in vivo.

MATERIALS AND METHODS

Determination of a complete HRSV subtype B genomic sequence directly from clinical material. Deidentified clinical material was kindly provided by Peter Coyle (Royal Victoria Hospital, Belfast, Northern Ireland). The sample was obtained from a tracheal rinse of an HRSV-positive infant during the 2004–2005 HRSV season (HRSV B05). Total RNA was extracted from clinical material (500 μl) using TRIzol LS reagent (Life Technologies). First-strand cDNA was generated using a SuperScript III first-strand synthesis system (Life Technologies) and negative-sense gene-specific primers based on conserved regions of the HRSV subtype B genome. PCR primers were designed to amplify the complete viral genome in six overlapping fragments. PCR was performed on the cDNA using Phusion High-Fidelity DNA Polymerase (New England BioLabs). PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced using primers spanning the viral genome. Sequences were assembled, and a consensus was determined using Lasergene, version 10 (DNASTAR). Primer sequences are available on request. rGHSV is a recombinant virus based on HRSV strain A2 (HRSV A2), which expresses GFP from an ATU present at position 1 (promoter proximal of the genome (19).

Construction of rHRSV B05 minigenomic and antigenomic plasmids. An HRSV minigenome plasmid, p(−)HRSV B05DI-EGFP, contained an EGFP open reading frame (ORF) flanked by the viral 3′ and 5′ termini and preceded upstream by a T7 promoter, guanine tri nucleotide, and ribozyme and followed downstream by a hepatitis delta virus ribozyme and T7 terminator sequences. A negative-sense viral RNA was produced upon transcription by T7 RNA polymerase. The minigenome construct was synthesized by GeneArt Gene Synthesis (Life Technologies) and ligated into a modified pBluescript vector (20). HRSV N, P, M2-1, and L expression plasmids were constructed in pCG(MPB) (21). A full-length, antigenic HRSV plasmid, pHRSVB05, was constructed following restriction enzyme digestion and sequential ligation into the modified pBluescript vector (20). The viral genome sequence was oriented with respect to the T7 promoter to produce an antigenomic RNA upon transcription. The full-length HRSV plasmid was modified to contain an ATU encoding EGFP located between the P and M genes, pHRSVB05EGFP(5).

Development of a minigenome assay and recovery of rHRSV. Confluent HEp-2 cells (ATCC, CCL-23) were infected with recombinant vaccinia virus MVA-T7 for 1 h at 37°C. Inoculum was aspirated, and Lipofectamine 2000 (Life Technologies) was used to transfect plasmid mixtures containing N, P, M2-1, L, and full-length or minigenome constructs. After 18 h the transfection mix was removed and replaced with OptiMEM (2 ml) (Life Technologies) containing 2% (vol/vol) fetal bovine serum (FBS). Cells were incubated for up to 7 days at 37°C with 5% (vol/vol) CO2. Supernatants from cells transfected with full-length constructs were used to infect fresh HEp-2 monolayers, and the presence of virus was confirmed by immuno-plaque assay, fluorescence, or CPE observed by phase-contrast microscopy. Cells, transfected with the HRSV minigenome construct, were observed daily by fluorescence microscopy to detect EGFP expression. Virus stocks were prepared in HEp-2 cells. Virus titers were determined by endpoint titration in HEp-2 cells.

In vitro infection assays and virus characterization. Growth kinetics was assessed by infection of HEp-2 cells at a multiplicity of infection (MOI) of 0.1. Triplicate samples were scraped, sonicated, and centrifuged to remove cellular fragments; the supernatant was snap-frozen as cell-free virus stock. Virus present in the sample for each time point was determined by endpoint titration in HEp-2 cells. Titers are expressed as 50% tissue culture infective doses (TCID50) calculated by the Reed and Muench method (22). To determine glycosaminoglycan (GAG) indices, CHO cells expressing GAG or GAG-deficient cells were infected and analyzed by flow cytometry (23). wd-NHBE cells were cultured in 12-mm/0.4-μm-pore-size inserts (Corning) at ALI (24). The apical surfaces of cells (estimated to contain 105 cells exposed at the surface) were infected at 25 to 26 days after growth at ALI. After 1 h of incubation at 37°C, inoculum was removed, and the apical surfaces were washed three times with Dulbecco’s phosphate-buffered saline (DPBS) (500 μl). At 2 days postinfection (d.p.i.), DPBS (500 μl) was added to the apical compartment, and the cells were incubated at 37°C. After 10 min the DPBS and growth medium were harvested from the apical and basolateral compartments, respectively, for virus isolation and quantitative PCR (qPCR). Subsequently, automated whole-well scans were made by confocal laser scanning microscopy (CLSM) with an LSM700 system fitted on an Axio Observer Z1 inverted microscope (Zeiss), followed by semiautomated enumeration of EGFP-positive (EGFP+) cells (DotCount; MIT, Boston, MA). Viruses were titrated in HEp-2 cells using 10-fold (growth kinetics) or 3-fold (apical rinse) dilutions in flat-bottom 96-well plates and cultured for 5 to 7 days at 37°C. The presence of HRSV genomes in samples was determined by TaqMan reverse transcription-PCR (RT-PCR) as previously described (25) with slight modifications. A quantified positive control for HRSV B (Vircell) was added to express the results in genome equivalents. The cycle threshold (Ct) value was calculated automatically when the fluorophore signal (6-carboxyfluorescein [FAM] for HRSV A and tetramethylrhodamine [TAMRA] for HRSV B) was detected above the background level and was used to give a quantitative indication of viral copy numbers. All in vitro experiments were performed at least three times. Statistical analyses were performed with SPSS, version 20.0.
HRSV immuno-plaque assay. Serial 10-fold dilutions of HRSV were prepared in OptiMEM. Confluent HEp-2 cells cultured in 24-well plates were infected with each dilution (200 TCID₅₀) for 1 h at 37°C. Incubation was aspirated, and 0.8% carboxy-methylcellulose (2 mL) (Sigma), in OptiMEM containing 2% (vol/vol) FBS, was added. Overlay medium was removed at 4 to 5 d.p.i., and cells were fixed in cold 80% (vol/vol) methanol for 1 h at 4°C. Plates were washed in distilled water and blocked with 5% (wt/vol) milk for 30 min. Goat anti-HRSV (Ab20745-1; Abcam) diluted 1:100 in blocking solution (200 μL) was added. Following 1 h of incubation at room temperature with rocking, plates were washed in distilled water, and rabbit anti-goat horseradish peroxidase (HRP) conjugate (Ab6741; Abcam) diluted 1:100 in blocking solution (200 μL) was added. Following 1 h of incubation at room temperature, plates were rinsed, and binding of the HRP-conjugated antibody was detected using 4-chloro-1-naphthol (200 μL), which was converted to produce a gray/black pigment (Pierce).

In vivo infection experiment. Six groups of six female, 3- to 4-week-old cotton rats were infected intranasally with 10⁴ TCID₅₀ of HRSV in an inoculum volume of 10 μL or 10 μL to target the URT or LRT predominantly (26). Animals (n = 3/group) were euthanized by exsanguination at 4 or 6 d.p.i. The right lung was inflated with 2% (wt/vol) agarose (Sigma-Aldrich), sliced, and submerged in medium (27). Postmortem nasopharyngeal washings were collected, and the left lung was prepared for PCR. Nasal concha, nasal septum, and aga rose-inflated right lung (27) were screened and scored for microscopic fluorescence (AxioVert 25; Zeiss), Mann-Whitney U tests were used to compare differences between groups, and a P value of ≤ 0.05 was considered statistically significant.

Immunohistochemical (IHC) analysis of formalin-fixed tissues. Paraffin-embedded tissues were processed as previously described (28). HRSV-infected cells were detected using a polyclonal rabbit antibody to EGFP (Invitrogen). All fluorescently stained slides were assessed, and digital fluorescent images were acquired with a Leica DFC digital camera using Leica Q500 software.

Confocal laser scanning microscopy. Nasal tissues and agarose-inflated lung slices were fixed with phosphate-buffered saline (PBS) containing 4% (wt/vol) paraformaldehyde, permeabilized with PBS containing 0.1% (vol/vol) Triton X-100 for 30 min, counterstained with the far-red nuclear counterstain TO-PRO3 (Invitrogen) or 4',6-diamidino-2-phenylindole (DAPI; Vectashield), and directly analyzed for EGFP fluorescence (Axiovert 25; Zeiss). Analyses were performed using Zen (Zeiss) or LCS (Leica) software.

Nucleotide sequence accession number. The complete genome sequence of HRSV B05 is available from GenBank under accession number KF640637.

RESULTS Generation of a wild-type, subgroup B BA rHR SV. Total RNA was extracted directly from a tracheal rinse sample obtained from an infant infected with HRSV, and high-fidelity RT-PCR and rapid amplification of cDNA ends (RACE) were used to generate ampiclons using previously described methods (29). The consensus genome sequence indicated that the virus belonged to the Buenos Aires (BA) genotype of HRSV subgroup B. This genotype was first detected in Argentina in 1999 and is characterized by a 60-kb deletion in the 5′-UTR (30). Virus loads determined by qPCR showed a good correlation with the numbers of EGFP⁺ cells. Neither released virus nor virus genome
was detected in the basolateral compartment (data not shown). This is consistent with the epitheliotropic nature of HRSV.

**rHRSV**B05 **efficiently infects cotton rats.** Cotton rats were infected intranasally with 10⁴ TCID₅₀ of rHRSV B05 or rHRSV B05EGFP(5) in a low volume (10 μl) to target the URT. Animals were sacrificed at 4 or 6 d.p.i., and unfixed respiratory tracts were screened by UV microscopy. High numbers of EGFP⁺ cells were detected at 4 d.p.i. in the nasal cavity of rHRSV B05EGFP(5)-infected animals (Fig. 3A). Discrete tracks of fluorescent cells were present in the epithelium of the nasal septum, reminiscent of what was previously observed in wd-NHBE cells (12). No EGFP⁺ cells were detected microscopically in trachea or lungs. Pathological assessment and immunohistochemistry (IHC) in 7-μm formalin-fixed lung sections indicated that both viruses predominantly infected ciliated respiratory epithelial cells (Fig. 3B) and caused destruction of the epithelium (Fig. 3C).

In order to target both the URT and LRT, cotton rats were intranasally infected with 10⁴ TCID₅₀ in a larger volume (100 μl) (26). Macroscopically, fluorescence levels in the nasal concha and nasal septum were indistinguishable between animals infected with the low- or high-volume inoculum, and no EGFP⁺ cells were

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**FIG 1** Development of a reverse genetics system for HRSV B05. (A) Schematic representation of HRSV eukaryotic N, P, M2-1, and L protein expression constructs and minigenome HRSV B05DI-EGFP showing the Le sequence, EGFP gene, and Tr sequence. (B) Phase-contrast and UV photomicrographs of HEp-2 cells at 2 days posttransfection with p(-)HRSV B05DI-EGFP and helper plasmids with (+L) and without (−L) the L protein expression clone. (C) Schematic representation of the rHRSV B05 genome. (D and E) Detection of the F glycoprotein of rHRSV B05 syncytia by indirect immunofluorescence. A negative control omitted the primary monoclonal antibody (E). (F and G) Detection of HRSV B05 by immuno-plaque assay. (H) Schematic representation of the rHRSV B05EGFP(5) genome. (I) Phase-contrast and fluorescent photomicrographs of rHRSV B05EGFP(5)-infected HEp-2 cells. (J) Growth curves determined by 10-fold titrations at four consecutive days on HEp-2 cells. (K) CHO cells expressing GAG and cells deficient in GAG were infected, and ratios were calculated and expressed as a GAG index. Data are presented as geometric mean titers ± standard errors. d.p.i., days postinfection; CMV, cytomegalovirus.
detected in the trachea. However, vastly different outcomes were observed when the lungs from infected animals were removed, inflated with agarose, sectioned, and screened for fluorescence. Infection with rHRSV<sup>Bos</sup>EGFP(5) resulted in high numbers of EGFP<sup>+</sup> cells at 6 d.p.i. in the epithelium of the bronchi and bronchioles in the lung slices (Fig. 3D). The number of EGFP<sup>+</sup> cells in the LRT was lower at 6 d.p.i.

Virus loads were determined in nasal lavage samples (Fig. 3E and F) and lung tissue (Fig. 3G and H) by virus isolation (Fig. 3E and G) or qPCR (Fig. 3F and H) for animals infected with a low (Fig. 3, hatched columns) or high (Fig. 3, nonhatched columns) volume of intranasal inoculum. The results corroborated the microscopic observations that high viral loads were detected in the URT of all animals (Fig. 3E and F) while only in animals inoculated with a high volume were significant virus loads detected in the LRT (Fig. 3G and H).

**Detection of rHRSV<sup>Bos</sup>EGFP(5) in the respiratory tract by optical sectioning.** The power of targeted pathology in understanding the spatial dynamics and pathological consequences of rHRSV<sup>Bos</sup>EGFP(5) infection is evident when standard IHC in the epithelium of the bronchi and bronchioles in the lung slices (Fig. 3D). The number of EGFP<sup>+</sup> cells in the LRT was lower at 6 d.p.i.

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**FIG 2** Infection of primary wd-NHBE cells grown at ALI with rHRSV<sup>Bos</sup> or rHRSV<sup>Bos</sup>EGFP(5). (A) Fluorescent photomicrographs and absolute counts of EGFP<sup>+</sup> cells/well for MOIs of 0.01, 0.1, and 1. (B) Virus isolations of corresponding apical rinses. (C) Genome equivalents in apical rinses measured by qPCR. Data are presented as geometric mean titers ± standard errors. ND, not determined.

**DISCUSSION**

We have developed a reverse genetics system based on an HRSV subgroup B clinical isolate and generated rHRSVs with or without an additional transcription unit encoding EGFP to study viral pathogenesis in the cotton rat model. Use of rHRSV<sup>Bos</sup>EGFP(5) allowed sensitive detection of infected cells both *in vitro* and *in vivo* in the early stages of infection in the absence of CPE. HEp-2 cells were suitable for virus passage *in vitro*, and the genomes were genetically stable: after 10 serial passages in HEp-2 cells, consensus sequencing revealed no mutations. In addition, the growth kinetics of rHRSV<sup>Bos</sup> and rHRSV<sup>Bos</sup>EGFP(5) were comparable, suggesting that insertion of an ATU into the HRSV genome did not result in virus attenuation. Laboratory-adapted viruses generated by extensive passage through a variety of disease-relevant and non-relevant cells and tissues have traditionally been used to develop molecular clones (34, 38–40).

HRSV spread in differentiated human airway epithelial (HAE) cells has been described as a “comet-like” spread, driven by the directionality of the beat of the cilia (12). Equivalent “comets” were present in the nasal conchae of infected cotton rats, demonstrating that these are relevant *in vivo* and not an *in vitro* artifact. Such localized virus spread has significant implications for the development and delivery of HRSV antivirals. We used the model to mirror the 1 to 2% of human cases where virus triggers bronchiolitis or severe pneumonia by varying the inoculation volume to target mainly the URT or concurrently the URT and LRT. Interestingly, rHRSV<sup>Bos</sup>EGFP(5) predominantly infected cells throughout the main branches of the bronchial tree, resulting in illumination of the bronchial tree. This aspect of HRSV pathogenesis has not previously been recapitulated in an animal model of HRSV or, to the best of our knowledge, in animals infected with any virus. Preferential infection of bronchial and bronchiolar epithelial cells mirrors the natural target cells of HRSV in humans (41). Moreover, at 4 d.p.i. rHRSV<sup>Bos</sup>EGFP(5) titers were similar to those obtained from patients or volunteers infected with HRSV (15, 16, 42). This illustrates the strength of the cotton rat model and shows the power of targeted pathology using EGFP-expressing recombinant viruses, which is only feasible due to the possibility of identifying infected tissues for blocking and processing immediately after necropsy.

Whereas existing *in vitro* and *in vivo* models of HRSV have focused mainly on subgroup A viruses, our recombinant virus is based on a subgroup B strain (31). Antigenic differences between the two subgroups of HRSV are predominantly mediated by the highly variable G gene (10) and might facilitate evasion of host immune responses (43). Despite these differences, infections with HRSV of either subgroup cause indistinguishable disease (1). Although outside the scope of understanding primary pathogenesis, this system should permit fitness experiments between viruses with and without the insertion in the G protein. This could explain why the BA viruses have outcompeted all other subgroup B HRSVs.

Reverse genetics of nonsegmented negative-strand RNA viruses has come a long way in the last 20 years following the recovery of rabies virus (44). The challenges of generating recombinant viruses are far from trivial, and much has been achieved with the original rHRSV systems (19, 34). Given the significant investment...
of time in establishing reverse genetics systems, there tends to be a large activation energy required to develop second- or third-generation systems. This is particularly true for HRSV, and, although tractable second-generation systems have been developed (45, 46), no group has successfully generated a virulent rHRSV fully reflecting the sequence of a current, clinically relevant, wild-type strain and studied primary pathogenesis in this key small-animal model. In addition, in vitro and in vivo models employing subgroup B HRSV strains have been scarce; these will be of crucial importance for preclinical testing of the effectiveness of new intervention strategies. It is vital to extend ongoing studies and move in the direction of reverse genetics systems based on clinical isolates grown in disease-relevant cells. Only then will it be possible to understand HRSV pathogenesis fully and systematically to test novel interventions. The recombinant B05 viruses will help in this endeavor, and these
should be augmented by the establishment of equivalent systems for subgroup A clinical isolates.

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