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Replacement of the F and G Proteins of Respiratory Syncytial Virus (RSV) Subgroup A with Those of Subgroup B Generates Chimeric Live Attenuated RSV Subgroup B Vaccine Candidates

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Human respiratory syncytial virus (RSV) exists as two antigenic subgroups, A and B, both of which should be represented in a vaccine. The F and G glycoproteins are the major neutralization and protective antigens, and the G protein in particular is highly divergent between the subgroups. The existing system for reverse genetics is based on the A2 strain of RSV subgroup A, and most efforts to develop a live attenuated RSV vaccine have focused on strain A2 or other subgroup A viruses. In the present study, the development of a live attenuated subgroup B component was expedited by the replacement of the F and G glycoproteins of recombinant A2 virus with their counterparts from the RSV subgroup B strain B1. This gene replacement was initially done for wild-type (*wt*) recombinant A2 virus to create a *wt* AB chimeric virus and then for a series of A2 derivatives which contain various combinations of A2-derived attenuating mutations located in genes other than F and G. The *wt* AB virus replicated in cell culture with an efficiency which was comparable to that of the *wt* A2 and B1 parents. AB viruses containing temperature-sensitive mutations in the A2 background exhibited levels of temperature sensitivity in vitro which were similar to those of A2 viruses bearing the same mutations. In chimpanzees, the replication of the *wt* AB chimera was intermediate between that of the A2 and B1 *wt* viruses and was accompanied by moderate rhinorrhea, as previously seen in this species. An AB chimeric virus, rABcp248/404/1030, which was constructed to contain a mixture of attenuating mutations derived from two different biologically attenuated A2 viruses, was highly attenuated in both the upper and lower respiratory tracts of chimpanzees. This attenuated AB chimeric virus was immunogenic and conferred a high level of resistance on chimpanzees to challenge with *wt* AB virus. The rABcp248/404/1030 chimeric virus is a promising vaccine candidate for RSV subgroup B and will be evaluated next in humans. Furthermore, these results suggest that additional attenuating mutations derived from strain A2 can be inserted into the A2 background of the recombinant chimeric AB virus as necessary to modify the attenuation phenotype in a reasonably predictable manner to achieve an optimal balance between attenuation and immunogenicity in a virus bearing the subgroup B antigenic determinants.

Respiratory syncytial virus (RSV) is an enveloped, negative-sense, single-stranded RNA virus that encodes 10 subgenomic mRNAs (7). Transcription of the viral genes is directed by short, conserved gene start (GS) and gene end (GE) *cis*-acting signals which flank each gene and are separated by intergenic regions with various numbers of nucleotides. These mRNAs are translated into 11 known proteins: 4 nucleocapsid proteins, namely, nucleocapsid (N) protein, phosphoprotein P, large polymerase subunit L, and transcription elongation factor M2-1; 3 transmembrane envelope glycoproteins, namely, fusion (F) protein, attachment (G) protein, and small hydrophobic (SH) protein; 2 nonstructural proteins, NS1 and NS2; the matrix (M) protein; and the RNA regulatory factor M2-2. The G and F glycoproteins are the major protective antigens and induce RSV-neutralizing antibodies and resistance to infection (7).

RSV remains the leading cause of serious viral lower respiratory tract disease in infants and children worldwide and accounts for approximately 90,000 hospitalizations in the pediatric population in the United States each year (7, 17, 28). The significance of RSV as a respiratory pathogen has made development of an effective vaccine a public health priority. We have focused on developing a live attenuated RSV vaccine which would be administered intranasally. This strategy, which mimics natural infection, has the advantage of inducing a balanced immune response that includes both serum and mucosal neutralizing antibodies and cytotoxic T cells (7). A second advantage of a live attenuated RSV vaccine is that such vaccines have not been associated with immune-mediated disease enhancement upon subsequent natural infection (26, 44, 45), whereas this has occurred with inactivated RSV vaccines (4, 24, 27).

Two antigenic subgroups of RSV, designated A and B, have been distinguished on the basis of antigenic and sequence dimorphism, which is observed across the genome but is most pronounced for the ectodomain of the G protein. Between subgroups, the G ectodomain differs at the amino acid level by up to 53% (23) and has 95% antigenic divergence based on reactivity of monospecific polyclonal sera (22). The mature F protein is 9% divergent by amino acid sequence (21) and 50% divergent antigenically between subgroups (22). Importantly,

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viruses of each subgroup are capable of causing severe lower respiratory tract infection, and prevention of serious RSV disease by vaccination will require a high level of protection against both subgroups. The prevalence of RSV subgroups A and B varies both between and within yearly outbreaks (19) and may alternate yearly (38). In addition, significant differences between the subgroups with regard to the age of infected children, gender, or frequency of nosocomial acquisition have not been observed (19). Recent vaccine studies of humans have provided evidence that 1- to 2-month-old seronegative infants, the target vaccine population, mount immune responses which are greater against the G protein than the F protein (43), and this G-protein-dependent immunity in very young infants would likely be greatly influenced by the antigenic differences between subgroups. These studies suggest that to achieve optimal immunization against RSV in the target population of very young infants, a bivalent vaccine containing components from both subgroups A and B will be required.

A number of live attenuated RSV vaccine candidates have been evaluated in animals and humans, and the most promising subgroup A vaccine candidates, based on vaccine candidate *cpts*248/404 and its recombinant counterpart rA2cp248/404, have been shown to possess both temperature sensitive (*ts*) and non-*ts* mutations which contribute to the attenuation phenotype (10, 16). For subgroup B, a comparable set of attenuated vaccine candidates or cDNA reagents from which to produce virus is not yet available (15, 25). An initial group of live attenuated subgroup B vaccine candidates was prepared by cold passage (*cp*) of RSV B1 (12). Two candidates from this group, *cp*-23 and *cp*-52, were shown to be attenuated in cotton rats; however, *cp*-23 and *cp*-52 appeared to be underattenuated and overattenuated, respectively, in human studies (25, 43). Fortunately, the use of reverse-genetic methods has provided an alternate strategy for the expedited development of vaccine candidates specific to RSV subgroup B. As shown in the present study, the F and G genes from wild-type (*wt*) strain A2 of subgroup A, and from a number of derivative vaccine candidates, were removed and replaced with the F and G genes from strain B1 of RSV subgroup B, resulting in a set of chimeric, recombinant AB viruses. We show that the chimeric *wt* AB virus replicates efficiently in vitro and in chimpanzees. We also show that chimeric AB vaccine candidates maintain the *ts* phenotype of the subgroup A virus background from which they were derived and that the attenuating mutations from subgroup A also attenuate the AB chimeric virus for chimpanzees. Thus, fully viable RSV AB chimeric vaccine candidates can be produced and further manipulated to achieve the desired level of attenuation appropriate for use in humans. The immunogenicity and efficacy of one particular AB chimeric vaccine candidate in chimpanzees indicates that it is ready for evaluation in humans.

MATERIALS AND METHODS

Cells and viruses. *wt* RSV strain A2 HEK-7 and *wt* RSV strain B1 were grown in HEp-2 or Vero cells as previously described (11, 13). For use in animal studies, viruses were diluted when necessary in Opti-MEM I (Life Technologies, Inc., Gaithersburg, Md.) immediately prior to use. The modified vaccinia virus Ankara recombinant expressing the bacteriophage T7 RNA polymerase (MVA/T7 pol) was provided by L. Wyatt and B. Moss and propagated in primary chicken embryo cells (46).

Assembly of chimeric clones. The *wt* RSV B1 genome has previously been sequenced in its entirety to establish a consensus sequence (GenBank accession no. AF013254). In order to prepare cDNA of the G and F genes, *wt* RSV B1 virus was grown in HEp-2 cell culture monolayers and concentrated from the clarified medium by precipitation with polyethylene glycol. The virion RNA was extracted and purified (40) and used as a template for reverse transcription (RT) with random hexamer primers (SuperScript Preamplification System; Life Technologies, Inc.). The resulting cDNA was subjected to PCR to amplify the G and F genes in a single cDNA. The PCR was performed with a positive-sense oligonucleotide designed to prime at the beginning of the intergenic region preceding the G gene, GCAT*GGATCC****TTAATTAA***AAATTAACATAATGAT GAATTATTAGTATG (the *Pac*I site, which occurs naturally in strain A2 at the SH gene end signal, is in boldface italics, a *Bam*HI site used for cloning the initial PCR product is in italics, and the subgroup B-specific sequence is underlined), and a negative-sense primer which hybridized midway through the intergenic region on the downstream side of the F gene, GTGTT*GGATCC*TGATT***GCAT GC***TTGAGGTTTTTATGTAACTATGAGTTAAG (annotated as described above, except that the *Sph*I site, which occurs in recombinant strain A2 as a marker introduced in the F-M2 intergenic region, is in boldface italics).

Four independently cloned G-F cDNAs were analyzed completely by nucleotide sequencing, and each was found to contain a similar error in the GE signal of G. To stabilize this region, a number of nucleotides were changed at the downstream end of the G gene and in the G-F intergenic region (Fig. 1). Mutagenesis was performed by a PCR-based procedure as previously described (3). This method involves two mutagenic oligonucleotides of positive and negative sense which abut one another and prime in opposite directions on the plasmid template. Following PCR, the mutagenized plasmid is ligated at the junction between the oligonucleotides, cloned, and analyzed for the correct sequence. The positive-sense oligonucleotide was as follows (broken into triplets at coding nucleotides, with nucleotide assignments which differ from the *wt* B1 sequence underlined, an introduced *Mfe*I site in boldface, and the G gene end and F gene start signals in italics): C-CAC-GCC-TAATG*AGTTA-TATAAAA***C AATT***G**GGGCAAATA*ACC-ATG-GAG. The negative-sense oligonucleotide was as follows (annotated as described above): GA-CTG-AGT-GTT-CTG-AG T-AGA-GTT-GGA-TGT-AGA-GGG-CTC-GGA-TGC-TG. The mutagenized *Pac*I-*Sph*I fragment, MH5-7, containing the G and F genes of subgroup B was sequenced in full and contained the correct sequence. This fragment was then cloned into the *Pac*I-*Sph*I sites of the following recipient cDNAs: D53 (*wt* A2), D53cp, D53ΔSH, D53cpΔSH, D53cp248/404ΔSH, and D53cp248/404/1030 (see footnote *a* to Table 1 for a description of the A2-derived mutations). These recipient cDNA clones all contain the 4C, nucleotide marker, and L gene restriction site mutations as previously described (42). This generated the following full-length viral cDNA clones for use in producing recombinant chimeric viruses: B/D53, B/D53cp, B/D53ΔSH, B/D53cpΔSH, B/D53cp248/404ΔSH, and B/D53cp248/404/1030.

Production of recombinant RSV. Transfection and recovery of recombinant RSV were performed as previously described (6). Briefly, HEp-2 cell monolayers infected at a multiplicity of infection of 3 with MVA/T7 pol were transfected with a full-length B/D53-based cDNA along with the pTM1-based N, P, L, and M2-1 expression plasmids by using LipofectACE reagent (Life Technologies, Inc.). Following 3 days of incubation at 32°C, clarified cell culture supernatants were passaged onto fresh HEp-2 cell monolayers that were incubated for 6 days at 32°C for the purpose of virus amplification. The virus was quantified by direct plaque titration with monoclonal antibody horseradish peroxidase staining as described previously (31). To ensure a homogeneous virus population, recombinant viruses were biologically cloned by three successive plaque purifications or terminal dilutions and then amplified two to three times on HEp-2 or Vero cell monolayers to produce virus suspensions suitable for characterization.

Virus characterization. The genomic RNA of each recovered virus was analyzed to confirm the presence of the G and F genes from B1 and the various *ts* and attenuation mutations from A2. This was done by RT-PCR and restriction enzyme analysis as previously described (40).

The *ts* phenotype of each recombinant virus was evaluated by determining the efficiency of plaque formation at various temperatures (14). Plaque titration was performed at 32, 35, 36, 37, 38, 39, and 40°C on HEp-2 cell monolayers incubated for 5 days in temperature-controlled water baths. The plaques were enumerated by antibody staining as indicated above.

The subgroup specificity of the expressed F glycoprotein was confirmed by immunostaining duplicate infected HEp-2 cell monolayers after 5 days with either F monoclonal antibody 92-11C (subgroup A specific) or 102-10B (subgroup B specific) (both generously provided by L. Anderson, Centers for Disease Control and Prevention, Atlanta, Ga.).

Studies with cotton rats. Replication of the chimeric and *wt* control viruses was evaluated in the upper and lower respiratory tracts of 6- to 8-week-old cotton rats (*Sigmodon hispidus*) maintained in microisolator cages. Groups of six cotton rats were anesthetized with methoxyflurane and inoculated intranasally with 10^6 PFU of chimeric or *wt* control virus in a 0.1-ml dose. Four days after inoculation, the cotton rats were sacrificed by CO_2 asphyxiation, and nasal turbinate and lung tissues were harvested separately for virus quantitation on HEp-2 cells, as described previously (12).

Studies with chimpanzees. Evaluation of the replication of chimeric *wt* virus rAB and chimeric attenuated virus rABcp248/404/1030 in the upper and lower respiratory tracts of chimpanzees was performed as previously described (9). Briefly, juvenile RSV-seronegative chimpanzees were anesthetized and inoculated by both the intranasal and intratracheal routes with 10^5 PFU of either virus in a 1.0-ml dose at each site. For virus quantitation following inoculation, nasopharyngeal-swab samples were collected daily for 12 days, and tracheal-lavage samples were collected on days 2, 5, 6, 8, and 12. Virus titers were determined for each sample by plaque assay on HEp-2 cell monolayers. The extent of rhinorrhea, a marker of upper respiratory tract disease, was estimated daily and as-

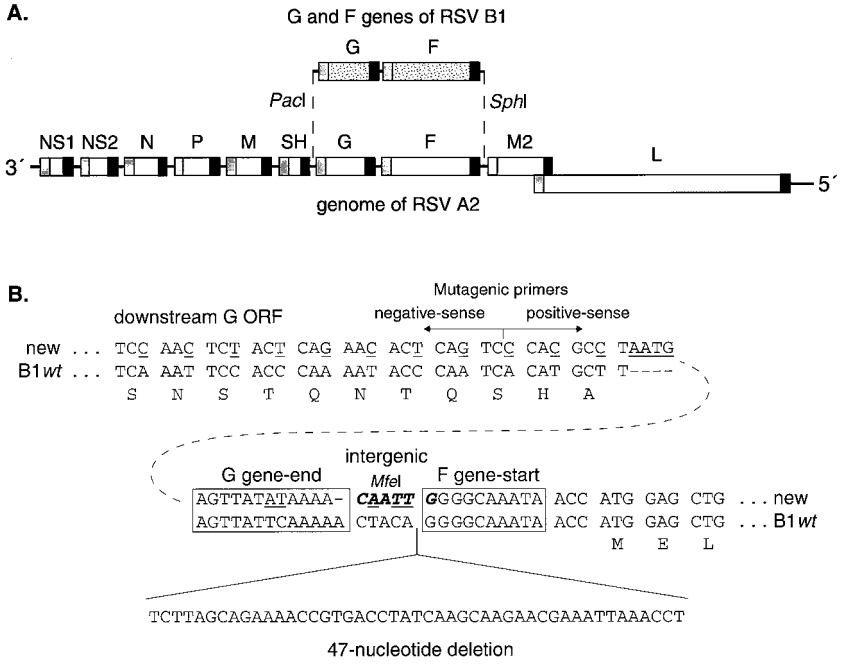

FIG. 1. Insertion of the F and G genes of RSV strain B1 into recombinant strain A2. (A) The G and F genes of RSV B1 (stippled rectangles) were amplified by PCR as a single cassette with oligonucleotide primers designed to create upstream *Pac*I and downstream *Sph*I restriction sites. Following an additional modification described below, this cassette was cloned into the naturally occurring *Pac*I site and the previously introduced *Sph*I site of the parental RSV A2 cDNA plasmid (6). The RSV genes are shown as open rectangles; the GS and GE transcription signals are shown as shaded and solid bars, respectively. (B) In order to stabilize the G gene end signal of the B1 cDNA, the sequence in this region was modified as shown (new): the third nucleotide in each of the last 11 codons of the G ORF was changed without altering the amino acid coding assignment; the termination codon of the G ORF was changed to an alternative termination codon; 4 nucleotides were introduced into the downstream nontranslated region of the G gene between the ORF and the gene end signal; the G gene end signal was made identical to that of the F gene of strain A2 by substituting 2 nucleotides and deleting a nucleotide in the A tract; and the G-F intergenic region was shortened by 47 nucleotides and an *Mfe*I restriction site was introduced. The sequence is divided into triplets at coding nucleotides, with the amino acid assignment shown directly below each codon. Nucleotides which differ from the B1 *wt* sequence are underlined, and the introduced *Mfe*I restriction site is shown in boldface italics.

signed a score of 0 to 4 (0, none; 1, trace; 2, mild; 3, moderate; and 4, severe). Twenty-eight days following inoculation, a pair of chimpanzees originally inoculated with rABcp248/404/1030 was challenged intranasally and intratracheally with 10^5 PFU of chimeric *wt* virus rAB in a 1.0-ml dose. Samples were collected as described above, and virus titers were determined.

RESULTS

AB chimeric RSV. We previously constructed antigenomic cDNAs encoding infectious recombinant *wt* RSV strain A2 (antigenic subgroup A) and derivatives containing various combinations of attenuating mutations. Here, we modified a panel of these A2 antigenomic cDNAs by replacing the F and G genes with their counterparts from the *wt* B1 strain of subgroup B. These were used to produce chimeric viruses, designated rAB, using the previously described transfection system (6). The recovered rAB viruses are represented by three groups: (i) *wt* virus rAB and its counterpart lacking the SH gene, rABΔSH; (ii) *cp* virus, rABcp, which contains the *cp* mutations found in the N and L genes of *cp*RSV (8, 42) (the two *cp* mutations in the F gene are lost by the gene substitution), and its counterpart lacking the SH gene, rABcpΔSH; and (iii) attenuated *cpts* viruses, rABcp248/404ΔSH, containing the *cp* and *ts* attenuating mutations of vaccine candidate *cpts*248/404 (10, 16) and lacking the SH gene, and the further-attenuated derivative rABcp248/404/1030, which in addition to the mutations of *cpts*248/404, contains a *ts* attenuating mutation derived from *cpts*530/1030 (41). By the use of RT-PCR and restriction fragment analysis, the chimeric viruses were confirmed to contain the G and F genes of subgroup B, as well as the appropriate SH gene deletion and *cp*, *ts*, or L gene restriction site mutations (data not shown). Together, this panel of viruses allowed the evaluation of the effect of the chimeric glycoproteins on *wt* RSV with and without the heterologous SH glycoprotein, their effect on temperature sensitivity, and their effect on virus attenuation in cotton rats and chimpanzees. The plaque size and yield of *wt* chimeric virus rAB was comparable to that of its *wt* subgroup A parent virus (data not shown). In addition, deletion of the SH gene recreated the previously reported effect of increased plaque size (2).

It was possible to transfer the complete F and G genes, rather than simply the open reading frames (ORFS), between viruses because the GS and GE signals are highly conserved between strains A2 and B1. Specifically, the 10-nucleotide GS signals of F and G are conserved exactly. The GE signal of G has three nucleotide differences (5′-AGTTACTTAAAAA for A2, with the underlined nucleotides being TTC for B1), all of which occur at positions whose assignments are variable among the RSV genes of both subgroups sequenced to date. The GE signal of F has a single-nucleotide difference (5′-AG TTATATAAAA for A2, with the underlined nucleotide being C for B1), which also occurs in a variable position. This amount of divergence for these signals between subgroups is no greater than that among the various genes within the A2 or B1 strain.

It should be noted that in initial work, the G gene cDNA of subgroup B was unstable during propagation in bacteria, such that the GE signal of G was elongated by the insertion of a long, variably sized tract of 30 or more A residues. It seemed likely that this occurred in response to incompatibility in bacteria of some unidentified sequence feature in this region of the cDNA. Retransformation and growth at a lower tempera-

TABLE 1. Temperature sensitivity and subgroup identity of *wt* rAB chimeric RSV and derivatives containing attenuating mutations[a]

Virus	Virus titer (log_{10}PFU/ml) on HEp-2 cells at indicated temp (°C)							Shut-off temp[b]	Reaction with MAb[c]:	
	32	35	36	37	38	39	40		92-11C	102-10B
rABcp248/404/1030	3.6	2.4[d]	<0.7	<0.7	<0.7	<0.7	<0.7	36	−	+
rABcp248/404ΔSH	5.2	3.9	3.8[d]	<0.7	<0.7	<0.7	<0.7	37	−	+
rABcpΔSH	4.6	4.6	4.8	4.8	4.4	3.6[d]	<0.7	40	−	+
rABΔSH	4.9	4.1	4.4	4.1	4.1	3.2[d]	<0.7	40	−	+
rABcp	4.8	4.0	4.4	3.8	3.2	3.2[d]	<0.7	40	−	+
rAB	4.5	4.3	4.1	4.2	3.1	3.7[d]	2.2	40	−	+
wt RSV A2	5.3	5.2	5.3	5.0	5.1	5.1	4.8	>40	+	−
wt RSV B1	4.9	4.9	5.0	4.6	4.4	3.8	3.2	>40	−	+

[a] The A2-derived mutations contained in the various rAB derivatives have been described previously and were derived from two different biologically derived viruses, *cpts*248/404 (16) and *cpts*530/1030 (41). The mutations were as follows: the *cp* genotype consists of five amino acid substitutions in the N (V267I), F (E218A, T523I), and L (C319Y, H1690Y) proteins, which are employed as a set and confer a non-*ts* attenuated phenotype (8, 42). Here, because of the replacement of the F gene, *cp* involves only the changes in N and L. The 248 and 1030 mutations involve amino acid substitution Q831L and Y1321N, respectively, in the L protein, each of which confers the *ts* and attenuation phenotypes (40, 41). The 404 genotype involves an amino acid substitution in L, D1183E, which is not associated with the *ts* or attenuation phenotype, and a nucleotide substitution in the GS signal of the M2 gene, with confers the *ts* and attenuation phenotypes (40). The ΔSH mutation involves deletion of the SH gene, which confers a modest attenuation phenotype in vivo (2).

[b] Shut-off temperature is defined as the lowest restrictive temperature at which a 100-fold or greater reduction of titer is observed. The virus titers at the shut-off temperature are boxed.

[c] Infected cell monolayers were immunostained with either RSV F monoclonal antibody (MAb) 92-11C (subgroup A specific) or 102-10B (subgroup B specific) and scored visually for staining. +, staining; −, nonstaining.

[d] Pinpoint plaque size.

ture and use of other bacterial strains failed to provide clones with the correct sequence at this signal. We therefore modified the GE signal and adjoining sequence (Fig. 1), which successfully stabilized the cDNA.

Temperature sensitivity and subgroup specificity of chimeric viruses. The level of temperature sensitivity of the chimeric viruses, as determined by their ability to form plaques at a range of temperatures, is presented in Table 1. All putatively non-*ts* chimeric viruses, including *wt* rAB, showed a slight temperature sensitivity, with little or no growth at 40°C, and pinpoint plaque sizes at 39°C. As expected, the addition of *ts* mutations to *wt* rAB increased its level of temperature sensitivity. The temperature sensitivity of chimeric virus rABcp248/404ΔSH (37°C shut-off temperature) was the same as that of its subgroup A counterpart rA2cp248/404ΔSH (39). Likewise, the temperature sensitivity of chimeric virus rABcp248/404/1030 (36°C shut-off temperature) was the same as that of its subgroup A counterpart rA2cp248/404/1030 (41). This showed that the *ts* mutations from subgroup A act independently of the surface glycoproteins expressed by the virus and that they can be used to attenuate the rAB virus in a reasonably predictable manner.

The subgroup specificity of the chimeric viruses was confirmed by reaction with subgroup-specific F monoclonal antibodies. As shown in Table 1, HEp-2 cell monolayers infected with subgroup A and B control viruses or each of the chimeric viruses were immunostained with subgroup A-specific (92-11C) and subgroup B-specific (102-10B) monoclonal antibodies. All chimeric viruses reacted only with the subgroup B monoclonal antibody.

Levels of replication of chimeric viruses in cotton rats. The levels of replication of *wt* A2, *wt* B1, and each of the chimeric viruses in the nasal turbinates and lungs of cotton rats following intranasal inoculation with 10^6 PFU of virus were determined (Table 2). Both *wt* control viruses grew to levels comparable to or greater than those previously reported (12). Surprisingly, the *wt* AB virus was restricted in growth compared to the two *wt* parents, with replication reduced approximately 6,500-fold in the nasal turbinates and more than 3,000-fold in the lung. An interesting observation in cotton rats is that the presence of the *cp* mutations appeared to significantly increase virus replication, especially for viruses lacking the SH gene (Table 2). Because of the replacement of the glycoproteins, the rABcp virus lacks the two *cp* mutations found in the F gene of subgroup A, which may be critical for the attenuation phenotype of the *cp* viruses. Nevertheless, the large disparity in replication capacity between the *wt* control viruses and each of the chimeric viruses suggested that in this case substitution of the F and G glycoproteins across subgroups was in and of itself strongly attenuating for cotton rats.

Levels of replication and immunogenicity of chimeric viruses in chimpanzees. The levels of replication of rAB and rABcp248/404/1030 in the upper and lower respiratory tracts of juvenile, RSV-seronegative chimpanzees were evaluated. Two chimpanzees were inoculated with *wt* rAB, and three were inoculated with one of the putatively attenuated chimeric viruses, namely, rABcp248/404/1030. Each virus was administered by intranasal and intratracheal instillation. Nasal-swab and tracheal-lavage samples were collected over the next 12 days, RSV titers were determined, and, because of the limited number of available animals, the results were compared to

TABLE 2. rAB chimeric RSV derivatives are attenuated in cotton rats

Virus[a]	No. of rats	Mean virus titer (log_{10}PFU/g of tissue)[b] (Duncan grouping)[c] in:	
		Nasal turbinates	Lung
A2 *wt*	6	6.48 ± 0.03 (A)	6.08 ± 0.06 (A)
B1 *wt*	6	6.43 ± 0.05 (A)	6.00 ± 0.05 (A)
rAB	5	2.62 ± 0.11 (B)	2.50 ± 0.27 (B)
rABΔSH	6	2.38 ± 0.24 (B)	2.21 ± 0.40 (B)
rABcp	6	3.48 ± 0.56 (C)	3.45 ± 0.23 (C)
rABcpΔSH	6	3.83 ± 0.11 (C)	4.00 ± 0.06 (C)

[a] Rats were administered 6.0 log_{10}PFU intranasally under light anesthesia on day 0 and sacrificed on day 4.

[b] Virus titer was determined in the nasal turbinate and lung tissues and is shown as mean titer ± standard error.

[c] Mean virus titers were assigned to statistically similar groups (A to C) by Duncan's multiple range test ($\alpha = 0.05$). Therefore, the means in each column with different letters are significantly different.

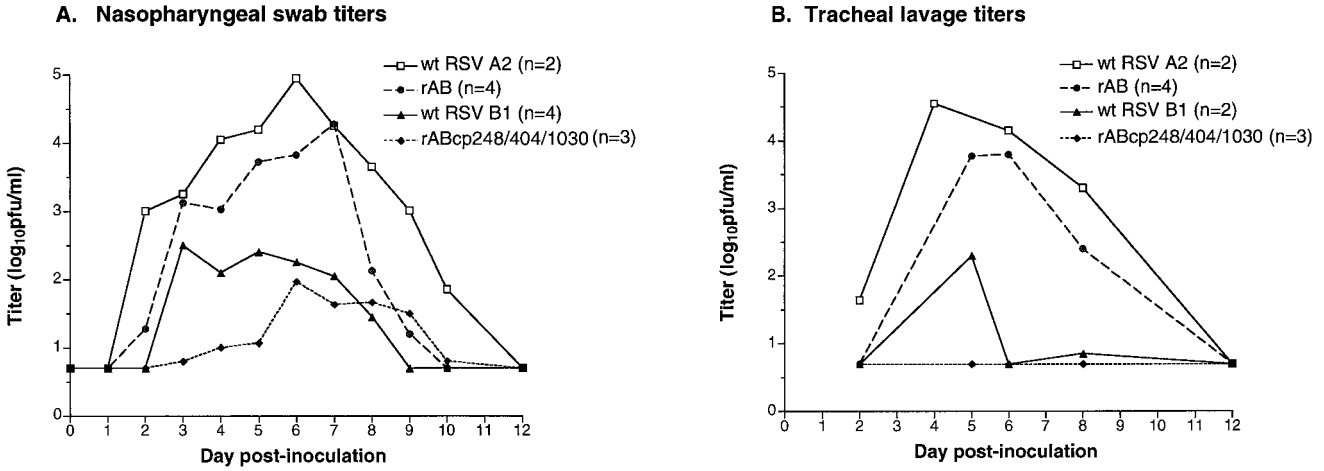

FIG. 2. Kinetics of replication in the upper (A) and lower (B) respiratory tracts of chimpanzees inoculated intranasally and intratracheally with *wt* control viruses A2 and B1 and chimeric viruses rAB and rABcp248/404/1030. The number of animals in each group (*n*) is shown. The dose of virus administered intranasally and intratracheally to each group was 5.0 $\log_{10}$ PFU/ml, except for *wt* RSV A2, which was administered at 4.0 $\log_{10}$ PFU/ml (11). Nasopharyngeal-swab and tracheal-lavage samples were collected on the days shown and titered by plaque assay on HEp-2 cell monolayers. The limit of detection for this assay is 0.7 $\log_{10}$ PFU/ml of sample. The data for *wt* RSV A2 and *wt* RSV B1 are from Crowe et al. (11, 12).

those of prior studies with *wt* A2 (13) and *wt* B1 (12). In both the upper and lower respiratory tracts, rAB replicated to levels that were somewhat lower than those of *wt* A2 but somewhat greater than those of *wt* B1 (Fig. 2). Thus, its chimeric nature seemed to be reflected in an intermediate level of growth. Specifically, in the upper respiratory tract, rAB exhibited a 30-fold increase in peak titer compared to *wt* B1 given at a comparable dose (Table 3). Likewise, in the lower respiratory tract, rAB exhibited a 40-fold increase in peak titer compared to *wt* B1 (Table 3). Chimeric virus rAB also induced a moderate level of rhinorrhea, comparable to that of *wt* B1. Although it was necessary to compare these results with those of historical control chimpanzees, this comparison helped demonstrate the *wt* nature of the rAB virus, making it suitable as a control virus for comparison with attenuated derivatives.

The chimeric virus rABcp248/404/1030 was highly attenuated in the upper and lower respiratory tracts of chimpanzees. Compared to rAB, this virus exhibited a nearly 200- and 2,000-fold decrease in peak titer in the upper and lower respiratory tract, respectively, and rhinorrhea was not observed (Table 3). However, even at this highly attenuated level of replication, rABcp248/404/1030 induced a significant level of serum neutralizing antibodies (Table 3) and protected the upper and lower respiratory tracts against subsequent challenge with *wt* virus (Table 4). This established the feasibility of attenuating the rAB virus by the insertion of A2-derived attenuating mutations into the A2 background. Furthermore, the high levels of attenuation and immunogenicity of the rABcp248/404/1030 virus identified it as a very promising vaccine candidate against RSV subgroup B.

DISCUSSION

Recombinant DNA technology has been used to create a number of chimeric RNA viruses, some of which represent novel vaccine candidates. Chimeric viruses have been generated in which regulatory regions, noncoding regions, or protein-coding regions have been exchanged between viruses. There are a number of reports of antigenic chimeric viruses, namely, ones in which one or more antigenic proteins have

TABLE 3. The *wt* rAB chimeric virus has a *wt*-like phenotype in chimpanzees and can be attenuated by inclusion of mutations derived from RSV subgroup A vaccine candidates

Virus[a]	No. of animals	Dose[b] ($\log_{10}$PFU per site)	Mean peak virus titer ($\log_{10}$ PFU/ml)		Rhinorrhea score[b]		Mean serum neutralizing antibody titer[c] (reciprocal $\log_2$)	
			Nasopharyngeal swab	Tracheal lavage	Peak	Mean	Day 0	Day 28
wt RSV B1	4[d]	4.0	2.98 ± 0.26	1.37 ± 0.67	3.0	2.5	<3.3	12.8
wt RSV B1	2[d]	5.0	2.75 ± 0.25	2.45 ± 1.45	2.0	1.5	<3.3	13.0
rAB	4	5.0	4.28 ± 0.12	4.05 ± 0.17	3.0	2.5	<3.3	12.3
rABcp248/404/1030	3	5.0	2.03 ± 0.19	<0.7 ± 0.00	0.0	0.0	<3.3	10.5

[a] Chimpanzees were inoculated by the intranasal and intratracheal routes with the indicated amount of virus in a 1-ml inoculum per site. Nasopharyngeal-swab samples were collected daily for 12 days, and tracheal-lavage samples were collected on days 2, 5, 6, 8, and 12.

[b] The amount of rhinorrhea was estimated daily and assigned a score (0 to 4) that indicated extent and severity: 0, none; 1, trace; 2, mild; 3, moderate; 4, severe.

[c] Serum RSV-neutralizing antibody titers were determined by a complement-enhanced 60% plaque reduction assay with *wt* RSV B1 and HEp-2 cell monolayer cultures incubated at 37°C (4). RSV-seronegative chimpanzee serum used as a negative control had a neutralizing antibody titer of <3.3 $\log_2$. Seropositive adult human serum used as a positive control had a neutralizing antibody titer of 11.0 $\log_2$.

[d] Historic control animals from Crowe et al. (12).

TABLE 4. Chimeric virus rABcp248/404/1030 is protective against challenge with rAB in the upper and lower respiratory tracts of chimpanzees

Immunizing virus[a]	Inoculum dose	No. of chimpanzees	Mean virus titer following RSV challenge (log_{10}PFU/ml)						Peak rhinorrhea score[b]
			Nasopharyngeal swab			Tracheal lavage			
			Day 3	Day 5	Day 7	Day 3	Day 5	Day 7	
rABcp248/404/1030	5.0	2	$<0.7 \pm 0.00$	1.00 ± 0.40	$<0.7 \pm 0.00$	$<0.7 \pm 0.00$	$<0.7 \pm 0.00$	$<0.7 \pm 0.00$	0
None		4	3.12 ± 0.16	3.72 ± 0.14	4.28 ± 0.12	$<0.7 \pm 0.00$	3.78 ± 0.26	3.80 ± 0.15	3

[a] Chimpanzees were inoculated by the intranasal and intratracheal routes with the indicated amount of virus in a 1-ml dose per site. After 28 days, the chimpanzees were administered 5.0 log_{10}PFU of rAB by the intranasal and intratracheal routes. Nasopharyngeal-swab and tracheal-lavage samples were then collected after 3, 5, and 7 days.

[b] The amount of rhinorrhea was estimated daily and assigned a score (0 to 4) that indicated extent and severity: 0, none; 1, trace; 2, mild; 3, moderate; 4, severe.

been replaced by their counterpart(s) from another virus, thereby combining the replicative proteins of one virus with the antigenic determinants of another. This has been done for a number of positive-sense RNA viruses: the structural proteins of flavivirus dengue type 4 were replaced with those from Langat virus (32) or from dengue virus type 1, 2, or 3 (1, 5); the envelope genes (prM and E) of yellow fever virus were replaced with the corresponding genes of Japanese encephalitis virus (30); the *env* gene of simian immunodeficiency virus was replaced with that from human immunodeficiency virus type 1 to create chimeric simian-human immunodeficiency viruses SHIVs (18); and the G-H loop in VP1 of foot-and-mouth disease virus serotype A was replaced with homologous sequence from serotype O or C (33). In addition, a variety of antigenic chimeras have been made in poliovirus (34). With the more recent development of reverse genetics methods for the nonsegmented negative-strand viruses, chimeric viruses have been made in which the glycoprotein of vesicular stomatitis virus (VSV) Indiana strain was replaced by that of the New Jersey strain (29), the H and F glycoproteins of measles virus were replaced by the G glycoprotein of VSV (36), and the HN and F glycoproteins of human parainfluenza virus type 3 (PIV3) were replaced with those of PIV1 (37).

Antigenic chimeric viruses can be appropriately attenuated for vaccine use in two ways. First, the combination of protective antigen genes from one *wt* virus with the remaining genes from a second *wt* virus can result in a chimeric virus whose proteins or sequence elements function inefficiently together, thereby resulting in attenuation. Following replacement of the H and F glycoproteins of measles virus by the G glycoprotein of VSV, the resulting chimeric viruses were restricted in cell culture up to 50-fold compared to their parent measles virus (36). Also, chimeric SHIVs have been shown to be attenuated yet immunogenic in macaque monkeys (18). There are also examples of chimeric viruses which are attenuated only by certain gene replacements: replacement of the dengue virus type 4 structural proteins with those of type 1 or 3 generated chimeric viruses that retained *wt* growth kinetics in simian cell culture and mouse neurovirulence (1, 5), while replacement with type 2 generated a chimeric virus that replicated more slowly than either parent virus and exhibited delayed neurovirulence in mice (1). Replacement of the prM and E genes of dengue virus type 4 with those from the tick-borne flavivirus Langat strain created chimeric viruses that showed a significant reduction in mouse neuroinvasiveness compared to the flavivirus parent (32). Second, the chimeric virus may be made with a virus recipient that has already been shown to be attenuated. Replacement of the HN and F genes of attenuated PIV3-cp45 with those from PIV1 generated a chimeric virus that maintained the level of attenuation of vaccine candidate *cp*45 yet provided immunization against PIV1 in hamsters (35). Similarly, replacement of the prM and E genes of the attenuated yellow fever virus vaccine with those from attenuated Japanese encephalitis virus created a chimeric virus that was shown to be more attenuated than either the yellow fever virus or the *wt* Japanese encephalitis virus yet immunogenic in rhesus monkeys (30). In this example, both components of the chimeric virus were derived from an attenuated virus, with the resulting antigenic chimeric virus being more attenuated than either parent virus.

Recently, the G glycoprotein of RSV subgroup B was expressed as an additional gene, rather than as a substitution, in RSV subgroup A (20). This virus therefore encodes two G proteins, one derived from subgroup A and one derived from subgroup B. Expression of this additional G glycoprotein attenuated the virus slightly in tissue culture; however, it is not known if the virus is attenuated in experimental animals. Since the F protein also exhibits significant subgroup specificity (21), it would be preferable to express both subgroup B glycoproteins in a subgroup B-specific vaccine. Toward this goal, in the present study the G and F glycoproteins of subgroup B were used to replace the G and F glycoproteins in *wt* or attenuated subgroup A viruses. To assess the feasibility of creating RSV AB chimeric viruses and the effect of chimerization on in vitro and in vivo replication of *wt* recombinant virus, the first virus generated was *wt* chimeric rAB. Next, chimeric viruses were made in a series of attenuated subgroup A virus recipients. These recombinant A2 recipients contained various combinations of attenuating mutations derived previously from strain A2 vaccine candidates (8, 39–42). These include the non-*ts cp* mutations, as well as the 248, 404, and 1030 *ts* attenuating mutations and the ΔSH gene deletion mutation (see footnote *a* of Table 1 for more details).

The AB chimeric viruses were evaluated with regard to their temperature sensitivity and plaque formation in vitro and their attenuation in cotton rats and chimpanzees. As shown in Table 1, the entire set of chimeric viruses, including the *wt* rAB chimera, was at least slightly *ts*, with a shut-off temperature of 40°C, even for the *wt* rAB chimera. However, this slight shift in *ts* phenotype due to chimerization did not augment the level of temperature sensitivity of rABcp248/404/1030 or rABcp248/404ΔSH, which had the same shut-off temperature as their subgroup A counterparts, rA2cp284/404/1030 and rA2cp248/404ΔSH (39, 41). Because the level of temperature sensitivity observed in the subgroup A vaccine candidates is reproducible in the AB chimeric viruses, the existing menu of *ts* mutations identified for subgroup A should be directly usable in the subgroup B chimeric vaccine candidates. The ΔSH mutation was of interest because SH is the third RSV surface protein and is of unknown function, and it was possible that it might interact with the F or G glycoprotein in a subgroup-specific manner. However, the presence or absence of the SH protein

of strain A2 did not affect the *ts* or attenuation phenotypes of the various rAB viruses.

The *wt* rAB chimeric virus showed a 1,000-fold reduction of replication in the upper and lower respiratory tracts of cotton rats, even though both subgroup A and B *wt* viruses replicated to very high titers in these animals. The other chimeric viruses also replicated at this lower level. This suggested that chimerization itself, i.e., the replacement of the F and G glycoproteins in RSV subgroup A by those of subgroup B, may have attenuated the *wt* chimeric virus. Since the *wt* rAB chimeric virus exhibited normal plaque size and replicated to *wt* levels in human HEp-2 cells, it was possible that the host range restriction of replication in the respiratory tract of the cotton rat would not be reflected in higher primates. We therefore evaluated the level of replication and immunogenicity of the wt rAB chimeric virus in chimpanzees. This virus replicated in chimpanzees to levels intermediate between *wt* B1 and *wt* A2 and caused rhinorrhea comparable to that of *wt* B1. This indicated that for the AB chimeric viruses, the cotton rat provides misleading information regarding attenuation for higher primates, since the *wt* chimeric virus replicates to a level approaching that of *wt* A2 in the more permissive chimpanzee model.

Having established the high level of replication of *wt* rAB in chimpanzees, it was possible to evaluate attenuated vaccine candidates, such as rABcp248/404/1030. This chimeric virus was highly attenuated in chimpanzees, as measured by the low levels of replication in the upper respiratory tract, the undetectable level of replication in the lower respiratory tract, and the absence of rhinorrhea. Nevertheless, rABcp248/404/1030 induced high levels of neutralizing antibody and provided a high level of protection from subsequent challenge with subgroup B virus.

Wright et al. recently evaluated the biologically derived *cpts*248/404 vaccine candidate in infants and children, including seronegative infants 1 to 2 months of age, who represent the target population for an RSV vaccine (43). This is the first RSV vaccine to be sufficiently promising to be tested in this young age group. This vaccine virus infected 84% of the recipients and induced detectable antibody responses in 82% of vaccinees, who were completely resistant to replication of a second, later vaccine dose, illustrating for the first time that an RSV vaccine can be effective in this age group. However, 71% of vaccinees experienced mild upper respiratory tract symptoms during the initial, but not subsequent, infection with the vaccine virus, indicating the need to further attenuate the *cpts*248/404 virus. This was recently accomplished in a recombinant version, called rA2cp248/404 (40), which was modified by the introduction of an additional mutation, namely the 1030 mutation, to create the further-attenuated derivative rA2cp248/404/1030 (41). The rABcp248/404/1030 virus that we describe here is the chimeric version of this virus. Both represent extremely promising vaccine candidates and will be evaluated clinically.

In summary, a live attenuated RSV vaccine should ideally be effective against both RSV subgroups. Our approach is to develop separate vaccine candidates effective against either subgroup A or B and then combine them into a single bivalent vaccine preparation. Presently, chimeric virus rABcp248/404/1030 and its subgroup A counterpart rA2cp248/404/1030 are being evaluated separately in humans. As part of our ongoing vaccine development program, additional subgroup A and B vaccine candidates are being prepared which lack the NS2 gene (39) and contain novel combinations of *ts* and attenuating mutations.

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