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The Temperature-Sensitive (*ts*) Phenotype of a Cold-Passaged (*cp*) Live Attenuated Respiratory Syncytial Virus Vaccine Candidate, Designated *cpts*530, Results from a Single Amino Acid Substitution in the L Protein

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***cpts*530, a candidate live-virus vaccine, is an attenuated strain of human respiratory syncytial virus (RSV). It was derived by subjecting a cold-passaged (*cp*) strain of RSV to a single round of chemical mutagenesis. *cpts*530 is a temperature-sensitive (*ts*) mutant that is attenuated in mice and chimpanzees, and its *ts* phenotype exhibits a high level of stability during replication in both species. In the present study, the complete nucleotide sequence of *cpts*530 RSV was determined. The five mutations known to be present in the parent *cp*RSV were retained in its *cpts*530 derivative, and one additional nucleotide change was identified at nucleotide (nt) 10060, which resulted in a phenylalanine-to-leucine change at amino acid 521 in the large polymerase (L) protein. To determine if this single amino acid substitution was indeed responsible for the *ts* phenotype of *cpts*530, it was introduced alone or in combination with the *cp* mutations into the full-length cDNA clone of the wild-type A2 RSV. Analysis of infectious viruses recovered from mutant cDNAs indicated that this single mutation specified complete restriction of plaque formation of recombinant *cp*530 in HEp-2 cell monolayer cultures at 40°C, and the level of temperature sensitivity was not influenced by the presence of the five *cp*RSV mutations. These findings identify the phenylalanine-to-leucine change at amino acid 521 in the L protein as the mutation that specifies the *ts* phenotype of *cpts*530. Furthermore, these findings illustrate the feasibility of using the cDNA-based recovery system to analyze and construct defined attenuated vaccine viruses.**

Human respiratory syncytial virus (RSV) is the most important viral agent causing bronchiolitis and pneumonia in infants and children (2), and a vaccine is needed to prevent the severe morbidity and significant mortality associated with infection by this virus. Since live attenuated RSV vaccines show considerable promise as agents for the control of this pathogen (2), we have been developing and characterizing such vaccine candidates (4, 5, 8, 10, 12, 14). The first live attenuated vaccine candidate was generated nearly 30 years ago by subjecting the wild-type subgroup A RSV strain A2 to 52 passages in bovine embryonic kidney (BEK) tissue culture at progressively lower temperatures, with the final temperature being 26°C (10, 12). This vaccine candidate, termed cold-passaged (*cp*) RSV, was neither significantly cold adapted nor temperature sensitive but was highly attenuated in seropositive adults and children, indicating that it possessed non-*ts* attenuating mutations. However, in seronegative children it still caused upper respiratory tract disease (10, 12). We sought to further attenuate the incompletely attenuated *cp*RSV by introducing *ts* mutations, which are known to attenuate viruses that infect the human respiratory tract and which can be readily monitored in tissue culture (17). *cp*RSV was mutagenized with 5-fluorouracil, and a series of *ts* progeny viruses was isolated, one of which was termed *cpts*530 (4–6). This mutant was 10-fold more restricted in its ability to replicate in the upper and lower respiratory tracts of mice than wild-type virus, and the *ts* phenotype was stable following prolonged replication in nude mice (5). *cpts*530 was attenuated, immunogenic, and phenotypically stable in seronegative chimpanzees, and infection of chimpanzees with *cpts*530 induced significant resistance to challenge with wild-type RSV. The *cpts*530 vaccine candidate was protective against challenge even if the chimpanzees were infused with RSV antibodies prior to the immunization, an experimental procedure that mimics the presence of maternal antibodies in young infants (6). We previously determined the complete nucleotide sequences of the genomes of *cp*RSV and two of its derivatives, *cpts*248 and *cpts*248/404 (3, 8, 9). In the present study, we extend this analysis by determining the complete nucleotide and predicted amino acid sequences of *cpts*530.

Recently, the technology to recover infectious virus from a cDNA clone of single-stranded, negative-sense RNA viruses, including RSV, was developed (1, 11, 13, 16, 18, 20). This means that the mutations occurring in an RSV vaccine candidate can be introduced individually or in combination into the full-length cDNA clone, and the phenotypes of the rescued recombinant viruses containing the introduced mutations can be determined. In the present study, the mutation unique to the *cpts*530 virus was introduced alone or in combination with the mutations unique to *cp*RSV to determine the genetic basis of the *ts* phenotype of *cpts*530.

MATERIALS AND METHODS

Cells and viruses. The RSV A2 wild-type and *cpts*530 strains were obtained and passaged on Vero or HEp-2 cells as previously described (3, 6, 10, 12).

Sequence analysis of *cpts*530. Total RNA was isolated from *cpts*530-infected Vero or HEp-2 cells with an RNeasy Total RNA Kit (Qiagen Inc., Chatsworth, Calif.) or TRIzol reagent (Life Technologies Inc., Grand Island, N.Y.), followed by phenol-chloroform extraction and ethanol precipitation. Reverse transcription (RT), amplification by PCR, cloning, and sequencing were performed as described previously, except that 10 overlapping PCR fragments were generated in the present study (3, 8, 9).

The nucleotide sequences of the 3′ and 5′ ends of the *cpts*530 mutant were

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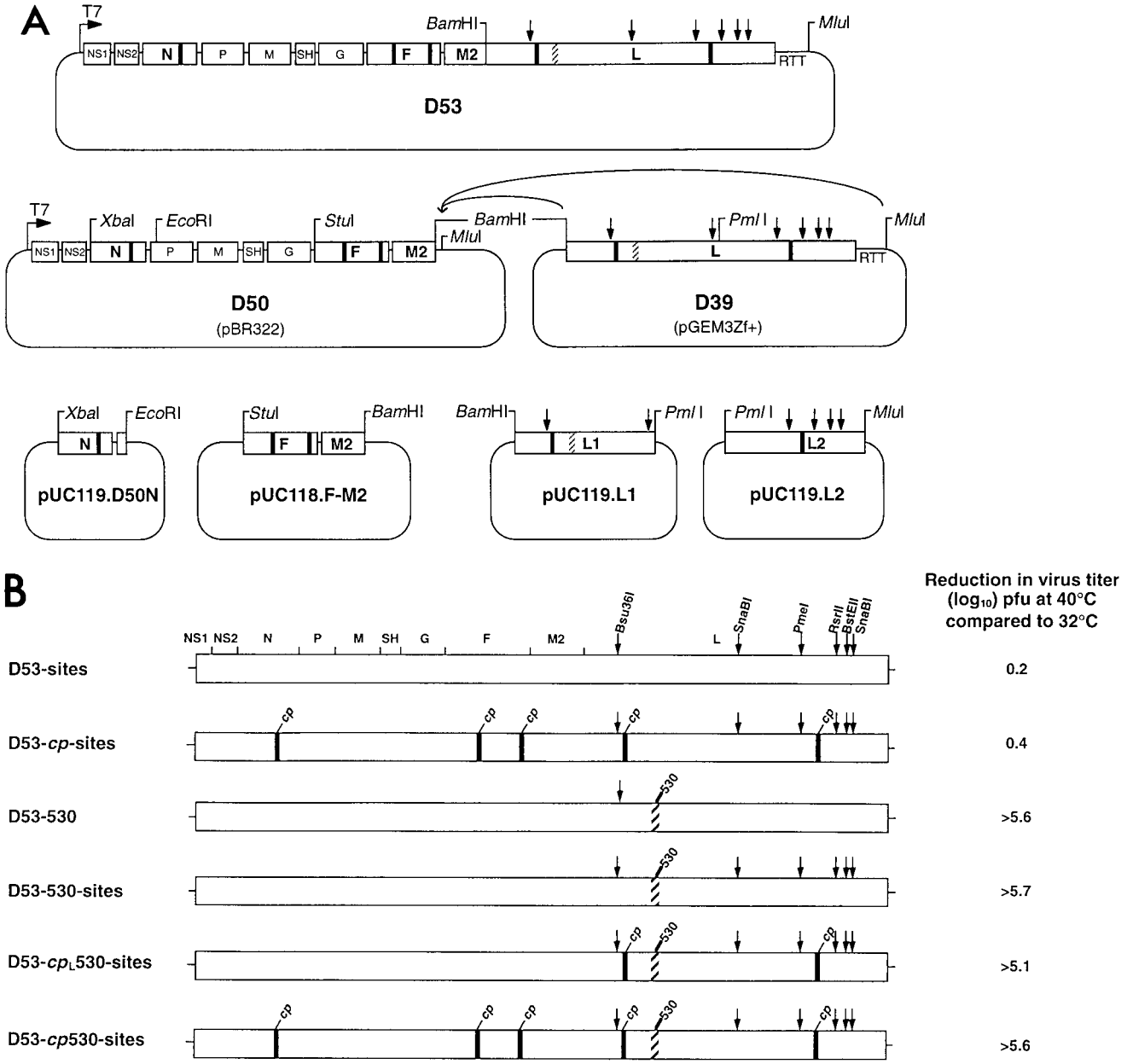

FIG. 1. Structures of cDNAs involved in the insertion of mutations, assembly of complete antigenome constructs, and recovery of recombinant virus. (A) Strategy for inserting mutations. Three types of mutations were inserted into the pUC118- or pUC119-borne cDNA subclones shown in the bottom row, namely, six silent restriction sites (arrows), five *cp* changes (solid bars), and the 530 mutation (hatched bar). The mutagenized subclones were inserted into the D50 (representing the RSV antigenome from the leader to the beginning of the M2-L overlap) or D39 (representing the RSV antigenome from the M2-L overlap to the trailer) intermediate plasmids shown in the middle row. The appropriate D50 and D39 plasmids were assembled into full-length D53 antigenome cDNA as shown in the top row (RTT indicates the hammerhead ribozyme followed by two T7 transcription terminators). (B) Maps of six mutant antigenome cDNAs which were used to recover recombinant RSV. The *ts* phenotypes of the recombinant viruses are summarized on the right based on data from Table 2.

determined as previously described (3, 9). Briefly, three clones of the 3′-end leader were prepared by polyadenylation of total RNA followed by RT-PCR. Ten clones containing the 5′-end trailer of the RSV genome were prepared from total RNA by RT, terminal transferase tailing, and PCR. All clones were manually sequenced with Sequenase version 2.0 (USB, Cleveland, Ohio).

Mutagenesis and assembly of full-length cDNA clones. The previously described RSV A2 wild-type full-length cDNA clone (1) had been designed to contain a single nucleotide insertion of C in the cDNA clone at nucleotide (nt) 1099 (which creates an *Afl*II site) and a total of six additional nucleotide substitutions at four loci. Thus, the nucleotide numbering system for the naturally occurring viruses and for the recombinant viruses derived from cDNA are out of register by 1 nt after position 1099. The nucleotide numbers in Table 1 are the positions for the naturally occurring viruses, while those for the cDNA clones and recombinant viruses derived from cDNA clones are 1 nt more (Table 2). One of the six nucleotide substitutions is a G-to-C change in genome sense at nt 4 in the leader sequence. This nucleotide variation has been detected in nonrecombinant RSV and was not found to have an effect on the temperature sensitivity of virus replication in tissue culture or in mice (9). Table 2 lists the various mutations which were inserted into the RSV cDNA in this study.

Intermediate clones (designated D50 and D39) were used to assemble the full-length RSV cDNA clone D53, which encodes positive-sense RSV antigenome (Fig. 1). The D50 plasmid contains the RSV genome from the leader to the M2-L overlap downstream of a T7 promoter, while the D39 plasmid encodes the full-length L gene and the trailer followed by the hammerhead ribozyme and two T7 terminators (approximately 7 kb long) bordered by *Bam*HI and *Mlu*I restriction sites. The full-length RSV cDNA clone (D53) used in transfections to

rescue infectious virus was assembled by inserting the *Bam*HI-*Mlu*I fragment of the D39 plasmid into the D50 plasmid (for detailed information, see reference 1).

Several pUC118- and pUC119-based constructs were derived from the D50 and D39 plasmids, and desired mutations were introduced into these constructs (Fig. 1A). Fragments containing the appropriate mutations were transferred from the pUC constructs back into the D50 and D39 plasmids as indicated in Fig. 1A, which in turn were assembled into a full-length clone. In this way, six different types of D53 full-length derivative clones were generated (Fig. 1B and Results).

Mutagenesis was performed by using the Muta-Gene Phagemid *in vitro* Mutagenesis Kit (Bio-Rad, Hercules, Calif.) as recommended by the manufacturer. The mutagenized constructs were transformed into competent *Escherichia coli* DH10B (Life Technologies Inc.). Miniprep DNAs from the transformant colonies were screened for the presence of the mutation by restriction enzyme digestion (see below) or by nucleotide sequence analysis of the mutagenized region.

Six translationally silent restriction site markers, the 530 mutation ($_{L521}$Phe→Leu), and the five *cp* mutations (see Tables 1 and 2) were introduced into the pUC-based constructs and subcloned into the D50 and D39 plasmids as described in the legend to Fig. 1A. The various full-length cDNA constructs were assembled by using D50 and D39 constructs containing different combinations of the above-mentioned mutations (Fig. 1B).

In the final cDNA constructs, the presence of the 530 and *cp* mutations were confirmed by sequence analysis, while the presence of the silent restriction sites was determined by restriction endonuclease analysis. Each D53-based construct was analyzed with various restriction enzymes (e.g., *Hpa*I, *Acc*I, *Hin*dIII, and *Pst*I), and the restriction patterns of the newly generated full-length cDNA clones were compared with that of the previously rescued wild-type full-length cDNA clone (data not shown). This restriction analysis was used to determine if an insertion or deletion of 100 nt or more had occurred during the bacterial amplification of the full-length plasmids.

Transfection and recovery of recombinant RSV. Transfection was performed as described previously (1). Briefly, monolayers of HEp-2 cells were infected at a multiplicity of infection of 1 with recombinant vaccinia virus MVA strain expressing T7 RNA polymerase (MVA-T7) and were transfected by using LipofectACE (Life Technologies Inc.) with a D53 antigenomic construct plus the N, P, L, and M2 (open reading frame 1) pTM1 support plasmids. On day 3, supernatants (clarified medium) were passaged onto fresh HEp-2 cells for amplification of rescued virus. Virus suspensions from this first amplification were harvested 5 days after infection and, following inoculation at various dilutions onto monolayers of HEp-2 cells, were overlaid with methylcellulose for plaque enumeration or with agarose for plaque harvest and biological cloning. Plaque enumeration was performed by a monoclonal antibody-horseradish peroxidase staining procedure as previously described (15). The recovered recombinant viruses were biologically cloned by three successive plaque purifications and then used to generate virus suspensions following two passages on HEp-2 cells. The biological cloning was important to ensure a homogeneous population of the recovered viruses, as recombination may arise during the first step of the rescue between the plasmid representing the full-length cDNA of RSV and the support plasmids containing RSV genes (11, 21). These biologically cloned and amplified virus suspensions were used in further molecular genetic or phenotypic characterization of the recombinant viruses. Two biologically cloned recombinant viruses were generated for each of the cDNA constructs (Fig. 1B) except for *cp*$_L$530-sites and *cp*530-sites, for which only one biological clone was generated. In each case, when there were sister clones, they were indistinguishable on the basis of genetic and biological analyses described below.

Genetic characterization of recombinant RSV. The recombinant RSVs generated as described above were characterized to determine if they did indeed contain each of the introduced mutations. Monolayers of HEp-2 cells were infected with biologically cloned recombinant virus, and total RNA was harvested 4 to 5 days postinfection as described above. RT was performed with random hexamer primers, and the generated cDNA was used as the template in PCR with the Advantage cDNA PCR Kit (Clontech Laboratories Inc., Palo Alto, Calif.) to generate three fragments representing almost the whole length of the recombinant RSV genomes. The PCR fragments corresponding to the RSV genome between nt 1 to 5131, 5949 to 10751, and 8501 to 15179. Also, a 544-bp fragment representing a portion of the L gene in the region of the 530 mutation between nt 9665 and 10209 was generated. This short PCR fragment was used in cycle sequencing (with a 71001 delta TAQ* Cycle Sequencing Kit [USB]) to confirm the presence or absence of the 530 mutation in the recovered recombinant virus, whereas the large PCR products were used in restriction enzyme digestion to confirm the presence of the silent restriction site markers and the *cp* mutations which were marked with specific restriction sites.

Characterization of the *ts* phenotypes of the recovered recombinant RSVs. Evaluation of the efficiency of plaque formation of the recombinant RSVs and the nonrecombinant control viruses was performed by plaque titration at 32, 37, 38, 39, and 40°C with HEp-2 monolayer cultures in temperature-controlled water baths as described previously (7, 9). Plaque identification and enumeration were performed by antibody staining as indicated above.

TABLE 1. Comparison of the complete nucleotide sequences of the RSV subgroup A wild-type (A2), *cp*RSV, and *cpts*530 viruses

Nucleotide position[a]	Amino acid position[b]	Gene	Nucleotide and amino acid change[c] in virus		
			A2 wt	*cp*RSV	*cpts*530
1938	267	N	G (Val)	A (Ile)	A (Ile)
6313	218	F	A (Glu)	C (Ala)	C (Ala)
7228	523	F	C (Thr)	T (Ile)	T (Ile)
9453	319	L	G (Cys)	A (Tyr)	A (Tyr)
10060	521	L	C (Phe)	C (Phe)	A (Leu)
13565	1690	L	C (His)	T (Tyr)	T (Tyr)

[a] Nucleotide positions are numbered from the 3′ end of the genome. The designated nucleotides are in the positive sense.

[b] Amino acid positions are numbered for each individual viral protein, starting from the AUG initiation codon.

[c] Boxes indicate the nucleotide changes between the wild-type (wt) A2 and *cp*RSV or *cp*RSV and *cpts*530.

RESULTS

Sequence analysis of *cpts*530. Ten overlapping PCR fragments representing the full-length genome of the *cpts*530 were cloned, and one clone of each fragment was sequenced entirely on both strands. Differences from the published sequence of the *cp*RSV parent virus (3, 9) were confirmed on both strands of a second clone, and, as needed, of a third clone. Additionally, the leader and trailer regions were each sequenced on one strand of a minimum of three independent clones. The sequencing results revealed that each of the five *cp* changes (3, 9) (Table 1) was conserved in the *cpts*530 mutant and one additional mutation at nt 10060 was identified (Table 1). This C-to-A change at nt 10060 resulted in the 530 mutation, a phenylalanine-to-leucine change at amino acid 521 in the L protein. Since *cp*RSV is not temperature sensitive, the findings summarized in Table 1 suggest that the amino acid substitution in L at amino acid 521 is responsible for the temperature sensitivity of *cpts*530.

Generation and characterization of recombinant RSV. We sought to address two questions related to the *ts* phenotype of *cpts*530. First, does the amino acid substitution in L that is unique to the *cpts*530 mutant indeed specify the *ts* phenotype? Second, is the level of temperature sensitivity specified by the 530 mutation influenced by the *cp*RSV mutations in the L, N, and F proteins? To address these questions, various combinations of mutations were introduced into the previously described cDNA encoding recombinant RSV (1). It should be noted that the recombinant virus generated in this initial study was found to be phenotypically indistinguishable from the biologically derived wild-type virus on the basis of its properties in tissue culture and its virulence in seronegative chimpanzees (1, 21). Thus, its cDNA is an appropriate substrate for introduction and analysis of putative *ts* and attenuating mutations. A total of six genetically distinct recombinant viruses were generated for analysis (Fig. 1B).

Six translationally silent restriction enzyme sites were introduced into the L gene, resulting in plasmid D53-sites (Fig. 1 and Table 2). These were designed to serve as genetic markers for identification of recovered virus and to facilitate cDNA manipulation. Also, it is known that vaccinia virus can mediate homologous recombination between an antigenome plasmid and support plasmids (11, 21). Since the L-gene support plasmid does not contain these markers, their presence in recov-

TABLE 2. Mutations introduced into the RSV A2 full-length cDNA clone

Type of mutation and gene[a]	Sequence of wild type[b]	Sequence of mutation[b]	Restriction site	Amino acid change
Site, L	$_{9398}$CTTAAGA	$_{9398}$*CCTAAGG*	*Bsu*36I	
	$_{11846}$TACATA	$_{11846}$*TACGTA*	*Sna*BI	
	$_{13339}$GTCTTAAT	$_{13339}$*GTTTAAAC*	*Pme*I	
	$_{14082}$CGTACAG	$_{14082}$*CGGACCG*	*Rsr*II	
	$_{14318}$TGTAACA	$_{14318}$*GGTAACC*	*Bst*EII	
	$_{14475}$TATGTA	$_{14475}$*TACGTA*	*Sna*BI	
cp				
N	$_{1935}$ATC**AGTT**	$_{1935}$*ATCGA*TT*	*Cla*I	$_{267}$Val→Ile
F	$_{6311}$TA**GAA**A	$_{6311}$*TCGC*GA*	*Nru*I	$_{218}$Glu→Ala
F	$_{7228}$**ACA**AAT	$_{7228}$*AT*TAAT*	*Ase*I	$_{523}$Thr→Ile
L	$_{9453}$**TGT***ATAC*	$_{9453}$**TA*C**ATAC	Lose *Acc*I	$_{319}$Cys→Tyr
L	$_{13555}$TATTAACTAAA**CAT**	$_{13555}$T*GTTAAC*TAAA**T*AC**	*Hpa*I	$_{1690}$His→Tyr
530, L	$_{10059}$**TTC**	$_{10059}$**TTA**		$_{521}$Phe→Leu

[a] The gene into which the mutation was introduced.
[b] Nucleotide differences between the wild type and mutants are underlined. Recognition sites of restriction endonucleases are in italic type. Codons in which the introduced nucleotide change(s) results in amino acid substitution are in bold type. The asterisk identifies the single nucleotide change that was present in the biologically derived *cp*RSV (Table 1). Numbering system reflects the 1-nt insertion in the full-length cDNA. The sequence is in the positive sense.

ered virus would confirm that the L gene is indeed derived from the antigenome cDNA. Virus recovered from this D53-sites cDNA clone would be expected to be ts^+ (i.e., wild type).

The D53-sites cDNA was further modified to contain the five amino acid substitutions of *cp*RSV (Table 2), resulting in D53-*cp*-sites. In the biologically derived *cp*RSV, each of the *cp* mutations involved a single nucleotide change (3, 9). The D53-*cp*-sites cDNA was designed so that four of the five *cp* coding changes involved two or three nucleotide substitutions relative to wild-type RSV (Table 2), with the idea that this would stabilize each mutation against reversion following replication in tissue culture and in vivo. Virus recovered from this cDNA would be expected to have the same phenotype as the biologically derived *cp*RSV, namely, ts^+ in tissue culture.

Additional antigenome cDNAs were made which contained the 530 mutation (Table 2) with various combinations of sites or *cp* mutations. The D53-530 cDNA contained the 530 mutation and only one of the six introduced restriction sites, whereas the D53-530-sites cDNA contained the 530 mutation and all six silent restriction sites. Based on the sequence analysis described above, recombinant viruses derived from either cDNA would be expected to be temperature sensitive. Also, we considered the possibility that one or more of the *cp* mutations are involved in determining the level of temperature sensitivity of *cpts*530. To address these questions, the D53-cp_L530-sites and the D53-*cp*530-sites plasmids were generated, containing two or five *cp* mutations, respectively (Fig. 1B).

Recombinant RSV was recovered from each of the six constructs indicated in Fig. 1B. Each recombinant virus was examined to confirm the presence of each introduced mutation. Total intracellular RNA from infected cells was used by RT-PCR to generate a PCR product representing the full-length L gene (between nt 8501 and 15179) of each recombinant virus (Fig. 2A shows the ~6.7-kb PCR product for each recombinant virus except for r*cp*-sites, which is not shown). The PCR products were gel purified and used in subsequent analysis with restriction enzymes to identify the presence or absence of the introduced mutations. The restriction analysis gave results as expected: r-sites, r530-sites, rcp_L530-sites, and r*cp*530-sites contained each of the silent genetic markers, while r530 contained only the expected *Bsu*36I restriction site. The results of this restriction enzyme analysis of the L-gene PCR product are shown in Fig. 2B for r*cp*530-sites. The presence of the *cp* mutations in rcp_L530-sites and r*cp*530-sites was confirmed by similar analysis using three PCR fragments corresponding to nt 1 to 5131, 5949 to 10751, and 8501 to 15179 of the RSV genome (data not shown). The presence of the 530 mutation in these recombinant viruses was confirmed by sequence analysis of a small fragment corresponding to nt 9665 to 10209. This is illustrated in Fig. 3 in which the sequence of r*cp*530-sites is compared with that of the wild-type A2 biologically derived virus.

Temperature sensitivity of recovered viruses. The levels of temperature sensitivity of the recombinant viruses and of the wild-type and *cpts*530 viruses is presented in Table 3. The following conclusions can be drawn from this information. First, the introduction of the silent restriction sites or the *cp* mutations does not confer a *ts* phenotype. This latter observation is consistent with our previous finding that the *cp*RSV is a ts^+ virus (5). Second, the 530 mutation does indeed specify the *ts* phenotype. Finally, the level of temperature sensitivity specified by the 530 mutation is not influenced by the *cp* mutations.

DISCUSSION

Nucleotide sequence analysis of the *cpts*530 virus identified a single mutation that is unique to this candidate vaccine virus, suggesting that this mutation specifies the *ts* phenotype. This finding was confirmed by the introduction of this 530 mutation into the full-length cDNA clone of the A2 wild-type ts^+ parent virus followed by the recovery of a *ts* recombinant virus bearing the 530 mutation. Analysis of the level of temperature sensitivity of this and additional recombinant viruses containing the 530 and *cp* mutations revealed that the level of temperature sensitivity specified by the 530 mutation was not influenced by the five *cp* mutations. Thus, it is reasonable to conclude that the 530 mutation alone specifies the *ts* phenotype and that it is also likely to be responsible for the small increment in attenuation in experimental animals manifested by *cpts*530 over that of its *cp*RSV parent (4, 6). The 530 mutation is a C-to-A change at nt 10060, resulting in a phenylalanine-to-leucine substitution at amino acid 521 in the L protein. This mutation

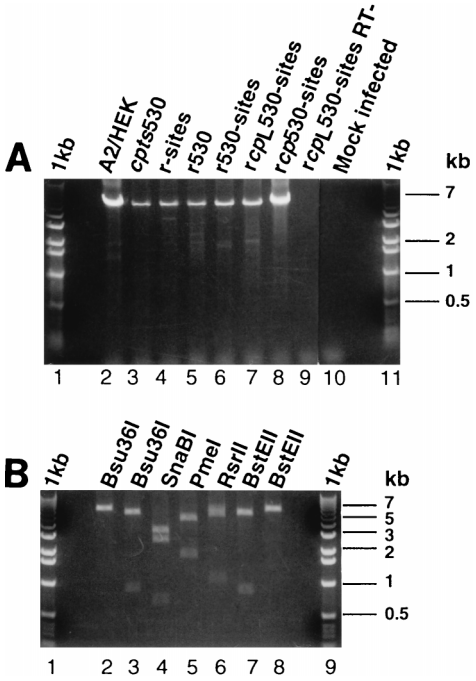

FIG. 2. RT-PCR amplification and restriction enzyme analysis of the L genes of recombinant viruses to confirm the presence of inserted mutations. (A) RT-PCR of infected-cell RNA was used to obtain cDNA of the complete 6.7-kb L gene of two biologically derived viruses, namely, type A2 (lane 2) and *cpts*530RSV (lane 3), and five recombinant viruses, namely, r-sites, r530, r530-sites, rcp_L530-sites, and r*cp*530-sites. Lane 9 shows the absence of a PCR product when the RT step was omitted; this particular sample was rcp_L530-sites RNA, and identical negative results were obtained for RNA from each of the other recombinant viruses (data not shown). Lane 10 shows the results of RT-PCR with RNA of a mock-infected culture. Lanes 1 and 11 contain size markers (1-kb DNA ladder; Life Technologies Inc.). The positions of the 0.5-, 1-, 2-, and 7-kb marker bands are indicated to the right of the gel. (B) The presence of inserted restriction sites was confirmed in r*cp*530-sites. The 6.7-kb L cDNA was generated as described above by RT-PCR of total RNA from cells infected either with biologically derived A2 wild-type virus (lanes 2 and 8) or recombinant virus r*cp*530-sites (lanes 3 to 7). Lanes 2 and 8 contain negative controls in which wild-type L cDNA was digested with *Bsu*36I or *Bst*EII restriction endonucleases, neither of which has a recognition site in the wild-type sequence. The 6.7-kb L cDNA of r*cp*530-sites was subjected to digestion with *Bsu*36I (expected sizes are 5.8 and 0.9 kb), *Sna*BI (expected sizes are 3.4, 2.6, and 0.7 kb), *Pme*I (expected sizes are 4.9 and 1.8 kb), *Rsr*II (expected sizes are 5.6 and 1.1 kb), and *Bst*EII (expected sizes are 5.8 and 0.9 kb). Lane 6 also contains a small amount of the original ~6.7-kb PCR product due to incomplete digestion with the restriction endonuclease *Rsr*II. Lanes 1 and 9 contain size markers (1-kb DNA ladder; Life Technologies Inc.).

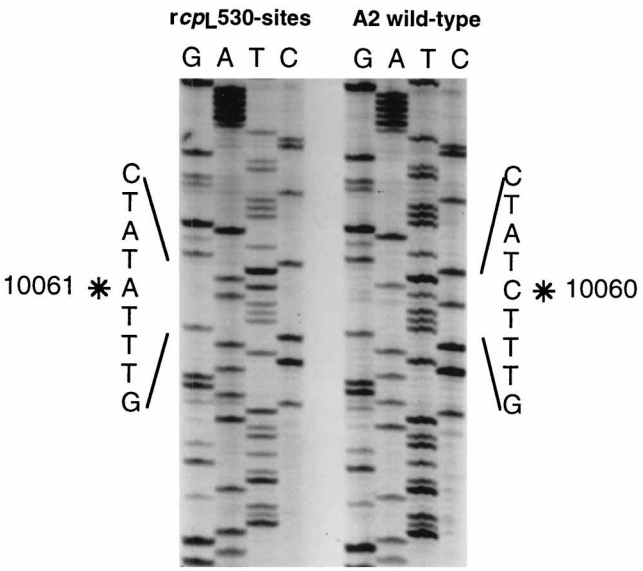

FIG. 3. Confirmation of the presence of the 530 mutation in recombinant virus r*cp*530-sites by nucleotide sequence analysis. A 544-bp fragment (nt 9665 to 10209) spanning the 530 mutation was generated by RT-PCR of infected-cell RNA and analyzed by cycle sequencing. A portion of the sequencing ladder containing the point mutation of interest (indicated by an asterisk and identified by its position in the complete sequence of the antigenome) is shown compared to the wild-type A2 virus. The sequence shown is in the positive sense.

causes the substitution of a nonpolar aromatic amino acid with a nonpolar aliphatic amino acid at a position which is well conserved (with >50% identity) among rhabdoviruses and paramyxoviruses and falls between the first and second conserved regions of the L protein (19). However, the biochemical basis of the temperature sensitivity specified by the 530 mutation is not known.

The findings from the present study have several important implications for the development of a live attenuated RSV vaccine. First, the insertion into and the recovery of mutations from the RSV A2 cDNA clone are relatively efficient. The antigenome cDNA clone used in this study had been modified in the original construction (1) to contain changes at five different loci, involving six nucleotide substitutions and one nucleotide insertion. The most extensively mutagenized virus described here contained mutations at an additional 12 loci involving 24 additional nucleotide substitutions. The fact that only the 530 mutation imparted a phenotype detectable in tissue culture indicates the relative ease of manipulation of this large RNA genome. Although recombination between the support plasmids and the full-length clone that is mediated by the vaccinia virus enzymes can occur (11), its frequency is sufficiently low that each of the 10 viruses analyzed here possessed the mutations present in the cDNA clone from which it was derived. Thus, it is now readily feasible to introduce attenuating mutations in a sequential manner into RSV to achieve the

TABLE 3. Comparison of the efficiency of plaque formation of recombinant and biologically derived viruses in HEp-2 cells at various temperatures[a]

Virus	RSV titer (log_{10} PFU/ml at the indicated temp)[b]				Reduction in virus titer (log_{10}) at the indicated temp compared to that at 32°C	
	32°C	38°C	39°C	40°C	39°C	40°C
Wild-type[c]	5.7	5.5	5.4	5.3	0.3	0.4
r-sites	5.4	5.1	5.2	5.2	0.2	0.2
r*cp*-sites	6.1	5.7	5.7	5.7	0.4	0.4
r530	6.3	6.0	4.4	<0.7	1.9	>5.6
r530-sites	6.4	6.1	4.4	<0.7	2.0	>5.7
rcp_L530-sites	5.8	5.9	3.8	<0.7	2.0	>5.1
r*cp*530-sites	6.3	5.0	4.1	<0.7	2.2	>5.6
*cpts*530[c]	6.6	5.8	4.4	<0.7	2.2	>5.9

[a] Efficiency of plaque formation of the various RSV strains was determined by plaque titration on monolayers of HEp-2 cells under semisolid overlay for 5 days at the indicated temperatures.

[b] Virus titers are the averages from two tests, except for r-sites and r*cp*-sites where data were derived from a single test.

[c] Biologically derived control viruses.

desired level of attenuation. Second, the present study unequivocally identified an attenuating mutation in the L gene, illustrating the use of the recovery system for direct identification of attenuating mutations. Previous findings from clinical studies and sequence analysis of the *cp*RSV virus suggest that the set of five non-*ts* mutations present in *cp*RSV are attenuating mutations for seropositive humans (3, 9, 10, 12). Our previous sequence analysis of the RSV A2 *cpts*248 mutant also identifies a single mutation in the L gene, a glutamine-to-leucine substitution at amino acid 831 (8), which is most likely to be attenuating and *ts*. Sequence analysis of *cpts*248/404 candidate vaccine virus revealed two mutations, a nucleotide change in the M2 gene start and an amino acid substitution, aspartic acid to glutamic acid at amino acid 1183 in the L protein (9). These mutations, if proven to be attenuating and/or *ts* using the analysis described here, can be assembled into a menu of attenuating mutations. These attenuating mutations, both *ts* and non-*ts*, then can be systematically introduced into the RSV A2 wild-type virus to produce a live attenuated virus that has achieved the proper balance between attenuation and immunogenicity. Third, it is advantageous that the 530 mutation and the other potential *ts* mutations identified to date are not in the G and F glycoproteins, which induce the protective immune responses to RSV in humans. This permits the development of an attenuated RSV cDNA backbone with mutations outside F and G that can serve as a cDNA substrate into which the F and G glycoproteins of RSV subgroup B or those of an epidemiologically divergent subgroup A strain can be substituted for the A2 F and G glycoproteins. In this way, a live attenuated RSV vaccine could be rapidly updated to accommodate antigenic drift within subgroup A strains and a subgroup B vaccine component could be rapidly produced. Fourth, we describe a strategy of constructing mutations to involve two or three nucleotide substitutions per codon rather than the one identified in the biologically derived mutant virus, a strategy that would be expected to improve genetic stability.

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