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Mutation of the C/EBP Binding Sites in the Rous Sarcoma Virus Long Terminal Repeat and *gag* Enhancers

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Several C/EBP binding sites within the Rous sarcoma virus (RSV) long terminal repeat (LTR) and *gag* enhancers were mutated, and the effect of these mutations on viral gene expression was assessed. Minimal site-specific mutations in each of three adjacent C/EBP binding sites in the LTR reduced steady-state viral RNA levels. Double mutation of the two 5′ proximal LTR binding sites resulted in production of 30% of wild-type levels of virus. DNase I footprinting analysis of mutant DNAs indicated that the mutations blocked C/EBP binding at the affected sites. Additional C/EBP binding sites were identified upstream of the 3′ LTR and within the 5′ end of the LTRs. Point mutations in the RSV *gag* intragenic enhancer region, which blocked binding of C/EBP at two of three adjacent C/EBP sites, also reduced virus production significantly. Nuclear extracts prepared from both chicken embryo fibroblasts (CEFs) and chicken muscle contained proteins binding to the same RSV DNA sites as did C/EBP, and mutations that prevented C/EBP binding also blocked binding of these chicken proteins. It appears that CEFs and chicken muscle contain distinct proteins binding to these RSV DNA sites; the CEF binding protein was heat stable, as is C/EBP, while the chicken muscle protein was heat sensitive.

Rous sarcoma virus (RSV) has strong transcriptional promoter and enhancer elements within the U3 region of its long terminal repeats (LTRs) (11, 26, 29; for reviews, see references 10 and 30). Once the viral DNA has been synthesized and integrated into the host DNA, viral RNA transcription is carried out by cellular RNA polymerase II and is controlled by other cellular factors (30). The 5′ terminal 100 nucleotides (nts) of the LTR contain enhancer activity; deletions in this region decrease LTR promoter function from 20- to 200-fold (11, 17, 26, 34). Additional enhancer activity has been detected intragenically within the *gag* protein coding region (3, 22, 47) and upstream of the 3′ LTR (26, 29).

The RSV LTR enhancer is transcriptionally active in many different cell types and in many different organisms (16). However, when avian retroviruses are injected into chicken embryos, highest levels of viral RNA expression are seen in skeletal muscle (14). Similar results were observed for expression of RSV LTR-driven chloramphenicol acetyltransferase (CAT) constructs in transgenic mice (36). Nuclear extracts from various avian cells have been shown to contain proteins binding to the 5′ enhancer region of the LTR; these were termed factors EFIIa, -b, and -c (45, 46); FI and FIII (15); a1/EBP (5, 39); and E2BP (24). In addition, a baby hamster kidney cell factor, IBF, was observed to bind to the *gag* enhancer sequences (23).

Our previous studies have shown that the purified CCAAT/enhancer DNA-binding protein (C/EBP) (18, 20, 27) binds to both the 5′ end of the LTR enhancer (nucleotides −225 to −188 of the SR-D strain) and the *gag* enhancer (nucleotides 813 to 872) of RSV and other avian retroviruses (7, 41). Heat-stable proteins in chicken liver nuclear extracts generate DNase I footprint patterns identical to those with C/EBP (7, 41). The *gag* enhancer has been localized between nts 788 and 1000, downstream from the transcriptional initiation site in RSV (7). While the LTR enhancer is considerably stronger than the *gag* enhancer, experiments with constructs containing both enhancers suggest they may act synergistically (3). We have previously derived a consensus core sequence for C/EBP binding ($T^T_G NNG^C_T AA^T_G$) and have shown that mutation of two of the conserved nucleotides is able to inhibit C/EBP binding (41). We have recently modified this consensus sequence to include additional DNA sequences shown to bind C/EBP avidly, and the extended consensus sequence we derived is

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A T   C  T
CT NNGAAA
G G   T  G
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(42). Note the high degree of degeneracy at several positions of this sequence and its tendency toward symmetry (ATTG CGCAAT).

C/EBP was purified as an enhancer core-binding protein for enhancers of simian virus 40, Moloney murine sarcoma virus, and polyomavirus and was expected to have a general tissue distribution (20). Instead, it appears to be restricted to terminally differentiated cells of fat, the liver, the intestine, the lung, the adrenal gland, and the placenta (4). Since RSV expression does not show a similar tissue distribution, it seems unlikely that C/EBP functions as a transcriptional activator of RSV. However, several proteins have been recently shown to compose a family of C/EBP-related proteins that are expressed in a variety of tissues (1, 6, 38, 48). These proteins display similar DNA binding specificities and share extensive amino acid sequence homology in the DNA binding region. Thus, there are a number of candidates for an activator of RSV transcription within this family.

In this study, minimal point mutations were introduced into the C/EBP binding sites within the LTR and *gag* enhancers and footprinting studies showed that these mutations eliminated C/EBP binding at these sites. These mutations reduced viral RNA levels as measured in both transient transfection and viral infection assays. In addition, we have detected proteins in chicken muscle nuclear extracts (CMNE) and chicken embryo fibroblast nuclear extracts

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(CEFNE) which bind RSV enhancers with the same sequence specificity as C/EBP.

MATERIALS AND METHODS

Construction of plasmids. pATV8K (9) contains the entire Prague strain, subgroup C (Pr-C), RSV genome (with a single LTR), permuted at the unique *Kpn*I site and inserted into pBR322. pAPrC (33) contains a nonpermuted Pr-C RSV genome with two LTRs. RSV sequence numbering is according to that of Schwartz et al. (44). mpSac9 (21) contains a *Sac*I fragment (RSV nts 6865 to 255) from pATV8K subcloned into M13mp18. p1Bam(+)f1 was constructed by insertion of the f1 intergenic region from pUCf1 (*Bam*HI-to-*Eco*RI fragment) (Pharmacia) into p1Bam(+) (7), which contains the RSV *Bam*HI fragment (nts 533 to 1917) downstream of the *cat* gene in pSV1cat (16).

Site-directed mutagenesis. The LTR mutations were made in mpSac9 (21), whereas p1Bam(+)f1 was the parental template for the *gag* enhancer mutant. Mutants PLC2, PLC12, and PGC23 were generated by the method of Zoller and Smith (49), and mutants PLC1 and PLC3 were generated by the method of Kunkel et al. (25). Mutations were confirmed by sequencing (43). C/EBP sites were numbered starting at the 5′ end of the LTR (41). Mutagenic oligonucleotides (underlined nucleotides indicate mutations) were PLC1, −212 5′GGAGTAGAGCATAAGAC3′ −228; PLC2, −194 5′GCATGTTTAAAGACTACAGG3′ −213; PLC3, −179 5′CTCATCGAAACATAAG3′ −194; and PGC23, 883 5′GGCTGTGGCACAGTTACAGCCTATAGCTGTACCA CAGTGATAGCAGG3′ 837. Oligonucleotides used for sequencing were complementary to LTR nts −141 to −125 and *gag* nts 927 to 942. The *gag* point mutations did not alter the amino acid sequence of the *gag* polyprotein.

DNase I footprinting. A modification of a protocol of Graves et al. (18) was used as described previously (41). Probes for the LTR footprints were obtained by labeling DNA with T4 polynucleotide kinase and [γ-^{32}P]ATP at the *Eco*RI sites of mpSac9 and its derivatives and then excising *Oxa*NI-*Eco*RI fragments (RSV nts 9014 to 9238), which were isolated from 2% agarose gels. An additional probe was generated by labeling at the *Oxa*NI site and excision with *Eco*RI. The *gag* enhancer probes were labeled at the *Nar*I sites (RSV nt 796) in pBam1(+)f1 and PGC23, and the fragments were excised with *Nde*I (nt 1290). C/EBP expressed in *Escherichia coli* was the generous gift of W. Landschultz and S. McKnight, and its preparation has been described previously (28). Before use, the lysate containing C/EBP was heated to 68°C for 10 min and chilled on ice, and denatured proteins were removed by centrifugation. CMNE were prepared from fresh chicken leg tissue (White Rock), which was the generous gift of Dover Poultry, Baltimore, Md. Extracts were prepared as described by Graves et al. (18). The yield from 220 g of tissue was 0.3 ml, containing 10 mg of protein per ml. CEFNE were prepared by a modification of a procedure described by Herget et al. (19). Primary cells prepared from 10-day-old chicken embryos were cultured in medium 199 with 2% tryptose phosphate broth, 1% calf serum, 1% chicken serum, penicillin, and streptomycin (all from GIBCO). The yield from 40 15-cm tissue culture plates of confluent cells was 2 ml of nuclear extract, containing 2.0 mg of protein per ml.

Transfection and RNase protection assay. CEFs were transferred on the day prior to transfection and were 70 to 90% confluent at transfection. A total of 10 μg of test DNA was transfected per 10-cm plate, together with 2 μg of pMyc23 DNA (32) as a control for transfection efficiency and RNA recovery. Test DNAs included the pATV8K (wild-type) clone of the RSV genome and site-directed mutants (KLC series), obtained by introducing the *Sac*I fragments from the mpSac9 constructs (PLC series) into pATV8K, as well as the pAPrC (wild-type) clone and the PGC23 *gag* mutant. Transfection was carried out in the presence of 200 μg of DEAE-dextran per ml, and cells were shocked for 3 min with 10% dimethyl sulfoxide at 5 h posttransfection as described previously (3). At 48 h posttransfection, RNA was harvested by the RNAzol (Tel-Test, Inc.) procedure (8). One-third of the RNA from each plate was used for each RNase protection assay. Viral RNA levels were assayed by an RNase protection assay with an antisense riboprobe containing Pr-C RSV sequences from *Bst*EII (nt 102) to *Bam*HI (nt 533) inserted into pGEM-2 as described previously (2). Expression from pMyc23 was detected with a riboprobe complementary to *myc* nts 2819 to 2923 in exon 2 (a gift from M. T. McNally). Overnight hybridization of the riboprobes to the test RNA was carried out at 55°C. RNase digestion of the hybridization reaction used 5 μg of RNase A per ml and 10 U of RNase T_1 (Calbiochem) per ml. Products were resolved on 6% denaturing acrylamide gels. RNA bands were quantified on a Molecular Dynamics PhosphorImager, and viral RNA levels were normalized relative to the levels of Myc23 RNA. Transfections were carried out in duplicate, and the average of four independent experiments was presented. Since the two different wild-type constructs expressed different levels of RNA, we normalized levels of expression for each mutant to that from the appropriate wild-type DNA.

CAT assays. Lysate from transfected CEFS was harvested 48 h after transfection. CAT assays were performed by the method of Gorman et al. (16). Results presented are the average of four independent experiments.

Reverse transcriptase assays. Transfection of pATV8K-derived DNA constructs was carried out as described above, with the modification that the DNA was cut with *Kpn*I (to remove the permuted viral insert from the vector) and ligated prior to transfection. The pAPrC constructs were transfected without prior cleavage. Virus was isolated from transfected cells after 11 days, and fresh CEFs were infected with equivalent reverse transcriptase units of the different viruses. Twelve days later, 50 μl of clarified supernatant from infected cells was assayed for reverse transcriptase as previously described (21). The results presented are the average of four independent experiments.

RESULTS

LTR mutations inhibited binding of C/EBP. Site-directed mutations were introduced into the C/EBP consensus sequences present in the LTR and *gag* enhancers. As shown in Fig. 1A, mutations introduced at each binding site altered two nucleotides in each of the three C/EBP binding consensus sequences previously identified in the Pr-C LTR by DNase I footprinting (41). Mutants were designated Pr-C LTR C/EBP site number (e.g., PLC1) (numbering of binding sites begins at the 5′ end of the LTR). To determine whether C/EBP binding had been affected by these mutations, DNase I footprint analysis was carried out with bacterially expressed C/EBP (28), and the results are shown in Fig. 2. The mutants with alterations in each of the three previously identified C/EBP binding sites and the double mutant (PLC12) all showed a loss of C/EBP binding over the

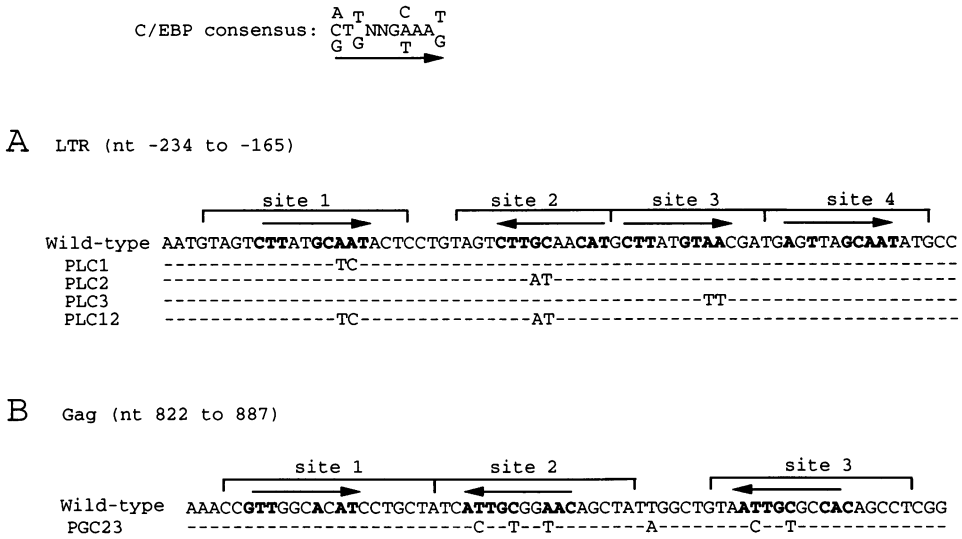

FIG. 1. Sequence of Pr-C LTR and *gag* enhancer C/EBP binding sites and their mutations. A modified consensus binding sequence for C/EBP (42) is shown at the top. (A) 5′ Terminus of the LTR from nts −234 to −165 showing the C/EBP binding sites detected as protected regions in DNase I footprint experiments. (B) Sequence of the *gag* enhancer in the region of the C/EBP binding sites (nts 822 to 887). Mutations introduced are shown in the sequence for mutant PGC23. Nucleotides conforming to the C/EBP consensus sequence are shown in boldface. Sequence alterations in the C/EBP site mutants are shown below the wild-type sequence. Dashes indicate nucleotides identical to those of the wild type. Arrows denote consensus sequence in either the sense (→) or the antisense (←) orientation.

mutated sites. This result verifies the importance of the core consensus sequence for binding of C/EBP.

In addition, these footprints revealed a fourth C/EBP binding site in the LTR at nts −167 to −181 relative to the transcription initiation site and adjacent to the 3′ end of the three sites previously observed (Fig. 2). The sequence of this binding site is shown in Fig. 1A (site 4); it shares 7 of 8 nts with the C/EBP consensus sequence. This site was not detected in the earlier series of footprint assays, which used smaller amounts of C/EBP (41).

C/EBP binding sites in the 3′ untranslated region of the RSV genome. The experiment whose results are shown in Fig. 2 also revealed binding of C/EBP at two sites upstream of the 3′ LTR in the 3′ untranslated region of the viral genome. These sites are designated PU1 and PU2 (Pr-C upstream site) (Fig. 2). This region of the Pr-C RSV genome (domain A, nts −303 to −234 relative to the start of transcription) has previously been shown to augment by about 10-fold the enhancer activity associated with the 5′ end of the LTR (domain B, nts −234 to −139) (26). Domains A and B together have a level of enhancer activity similar to that observed with the complete LTR enhancer in the absence of domain A (nts −234 to −53) (26).

To more precisely localize these upstream binding sites, we used a different probe labeled closer to the binding sites. This footprint (Fig. 3) allowed localization of the protected sequences: PU1, −306 TCTGATGGCCAAATAAGGCA −287, and PU2, −271 TGTAACTGCGAAATACGCTT −252, relative to the transcriptional initiation site at +1. Each of these sequences contains a 7-of-8-nt match to the consensus C/EBP binding site on the antisense strand (consensus underlined above). Previous DNase I footprint studies using smaller amounts of C/EBP detected only the LTR binding sites 1 and 2 strongly and site 3 weakly (41). Thus, it appears that LTR sites 1 and 2 may have a higher affinity for C/EBP than do either sites 3 and 4 or the upstream sites (PU1 and PU2). Interestingly, LTR sites 1 and 2 have an 8-of-8 match to the consensus C/EBP binding site, while the other sites observed have a 7-of-8 match (Fig. 1).

Since the enhancer activity in the region upstream of the 3′ LTR appears to be dependent on the strain of RSV assayed (26), we examined the same region of SR-D RSV (which differs considerably in nucleotide sequence [11]) for binding of C/EBP but failed to detect any binding (data not shown). In addition, we observed that SR-D, like Pr-C, possesses C/EBP binding site 4 (40), although it lacks binding site 3 because of a 5-nt deletion in this region of the LTR relative to Pr-C (12).

Mutagenesis of C/EBP binding sites within the *gag* enhancer. C/EBP binding sites within the *gag* enhancer region were similarly altered by site-directed mutagenesis (Fig. 1B). A single mutant which altered nucleotides in the consensus sites previously observed within this binding domain was created (7). Much of the DNaseé I footprint was abolished in the mutant; however, a protected region remained at the 5′ end of the binding site (Fig. 4). This region (Fig. 1B, site 1) has a 6-of-8 match to the C/EBP consensus sequence that may be mediating the residual C/EBP binding. This observation was unexpected since several mutations that generated 6-of-8 matches to the consensus failed to bind C/EBP (Fig. 2). The footprint and the nucleotide sequence suggest that the *gag* C/EBP binding region comprises three separate binding sites, each containing its own consensus: site 1, nts 825 to 843; site 2, nts 844 to 860; and site 3, nts 867 to 884. We termed the mutant PGC23 (Pr-C *gag* C/EBP sites 2 and 3).

In summary, we have observed four adjacent C/EBP binding sites at the extreme 5′ end of the Pr-C RSV LTR; three of these same sites were seen in the SR-D LTR. In addition, two sites specific to Pr-C were observed in the 3′ untranslated region upstream of the 3′ LTR. Further, three C/EBP sites were observed in the *gag* enhancer region; the sequence of this region is highly conserved among avian retroviruses. A schematic drawing with the location of the

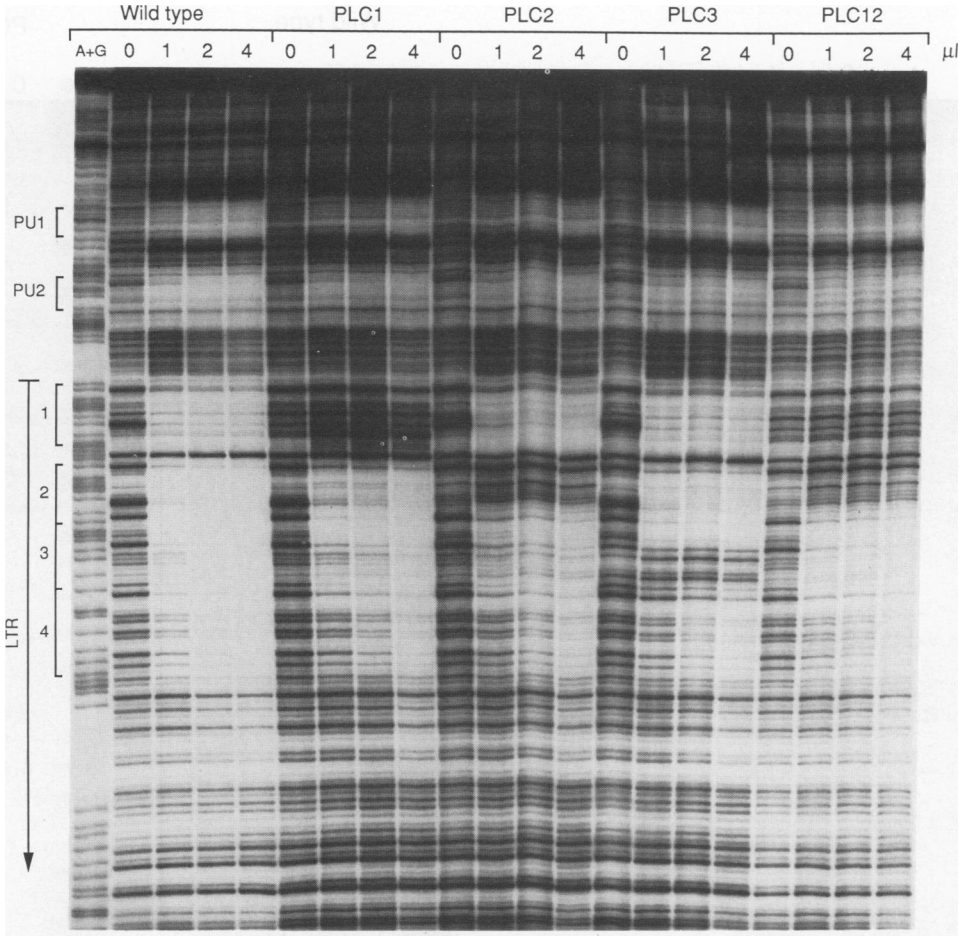

FIG. 2. DNase I footprint of wild-type and C/EBP site mutants of the Pr-C RSV LTR. Lane A+G is a Maxam and Gilbert sequence reaction (31) of the wild-type probe. Amounts of C/EBP in each reaction lane are shown at the top (in number of microliters of a 3-mg/ml stock). C/EBP binding sites in the LTR (sites 1 to 4) and in the 3′ viral untranslated sequences (PU1 and -2) are denoted at the left of the figure. DNA probes were 5′ end labeled at the *Eco*RI site at nt −54.

C/EBP binding sites we have mapped on the RSV genome is shown in Fig. 5.

Heat-sensitive factors in CMNE bind the C/EBP sites in RSV DNA. Since the tissue-specific expression pattern of C/EBP is different from that of RSV, we examined the binding of nuclear proteins from chicken muscle and CEF cells to the wild-type and mutant RSV DNAs. CMNE produced a DNase I footprint pattern (Fig. 6) that is identical to that observed with heat-treated chicken liver nuclear extract (41). On wild-type sequences of the Pr-C LTR, sites 1, 2, and 3 were protected and site 4 was not. This result was expected, since binding at site 4 was observed only with high concentrations of bacterially expressed C/EBP (Fig. 2). To compare the heat stability of C/EBP with that of the CMNE binding factor, we heated the CMNE at 68°C for 10 min and repeated the footprint. To our surprise, all DNase I protection of sites 1, 2, and 3 was eliminated by heating, suggesting that the CMNE protein binding these sites was heat sensitive (Fig. 6).

To further examine the DNA sequence specificity of CMNE factors, this extract was used to footprint DNA from the LTR of the mutants PLC1, PLC2, and PLC12 described above. As was seen with C/EBP (Fig. 2), mutation of the consensus C/EBP binding site blocked binding of proteins in the CMNE (reference 40 and data not shown), indicating that the sequence specificity of the muscle protein is similar to that of C/EBP. These results suggest that the binding activity of CMNE to the RSV LTR can be distinguished from that of C/EBP by its heat sensitivity but not by its DNA sequence specificity.

CEFs contain heat-stable binding proteins with sequence specificity identical to that of C/EBP. To further test the relevance of the C/EBP binding sites for RSV replication, nuclear extracts were prepared from cultured CEFs in which virus replicates readily. When binding of CEFNE proteins to wild-type LTR sequences was assayed by DNase I protection, the entire LTR region assayed (not just the C/EBP sites) was protected (data not shown). Similar footprints were previously obtained with unfractionated chicken liver nuclear extracts (40). After the CEFNE were heat treated, the DNase I protection pattern was restricted to C/EBP binding sites 1, 2, and 3 (reference 40 and data not shown) and showed a pattern of DNase I protection like that of unheated CMNE. When the LTR mutants PLC1, PLC2, and PLC3 were footprinted with the heat-treated CEFNE, decreased protection of the mutated sites was observed just as had been seen with C/EBP (reference 40 and data not shown). Thus, CEFs have nuclear proteins which bind the

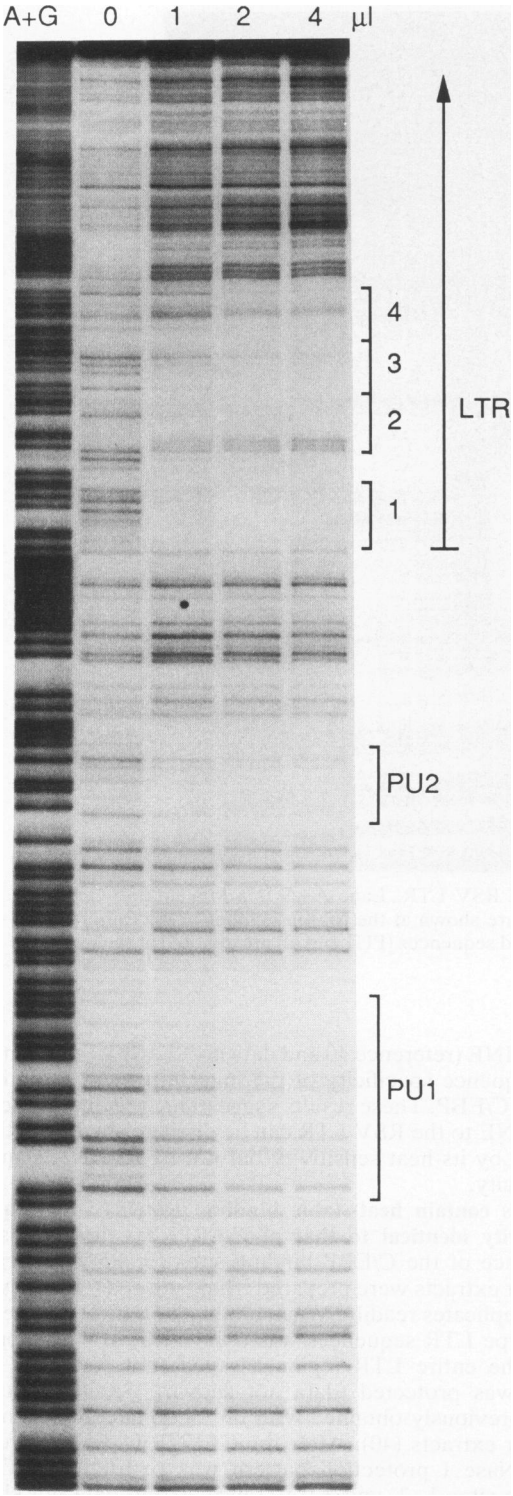

FIG. 3. DNase I footprint of the 3′ untranslated viral sequences upstream of the 3′ LTR. The DNA probe was labeled at the *Oxa*NI site. At right, brackets indicate the two C/EBP binding sites (PU1 and PU2). Also evident are the C/EBP binding sites within the LTR. For lane identifications, see the legend to Fig. 2.

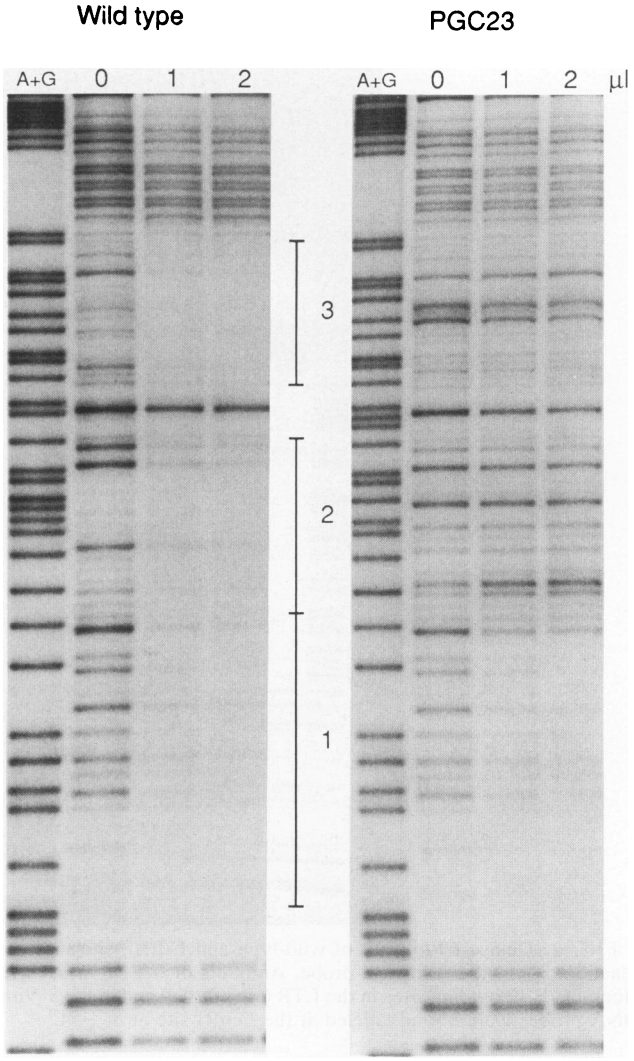

FIG. 4. Footprint of the C/EBP binding domains within the *gag* enhancer and the mutant with alterations in sites 2 and 3 (PGC23). Volumes of C/EBP used in the footprinting reactions are indicated at the top of the gel. Brackets between the panels indicate the C/EBP binding sites 1 through 3. For lanes A+G, see the legend to Fig. 2.

same DNA sequences as C/EBP and which also are heat stable, as is C/EBP.

LTR and *gag* enhancer mutations decrease viral RNA levels. In order to assess the significance of the different C/EBP binding site sequences in RSV DNA for viral replication, we introduced mutant viral DNAs into CEFs and measured RNA and virus production. The mutated LTRs were inserted into a permuted RSV construct containing a single LTR, pATV8K (9). These DNAs were transfected into CEFs, and total cellular RNA was prepared 48 h later and assayed for viral RNA levels by RNase protection (Fig. 7; Table 1). All of the LTR mutations led to some decrease in viral RNA levels. As expected, the site 1-plus-site 2 double mutation had the greatest effect, reducing RNA to an average of 42% of wild-type levels in transient assays. In the case of the single mutations, mutation of either site 1 or site 2 had a greater effect on RNA levels than did mutation of site 3 (Table 1). We conclude that the combination of LTR sites 1

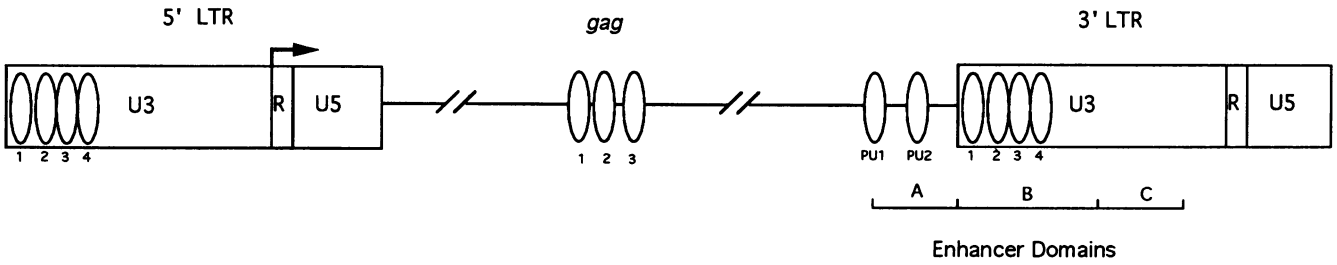

FIG. 5. Summary of binding sites for C/EBP and related proteins that have been mapped on the Pr-C RSV genome. The binding sites are indicated by ovals. A, B, and C represent the three enhancer domains described by Laimins et al. (26).

and 2 appears to be the primary element needed to achieve high RNA levels.

In these permuted pATV8K-derived constructs, the enhancer of the solo LTR is adjacent to the untranslated sequences at the 3′ end of the genome, which include an enhancer domain identified by Laimins et al. (26) that we have shown to bind C/EBP (Fig. 3). Thus, it was possible that the effects of the LTR mutations were partially obscured by the presence of this upstream element. We consider this to be unlikely since these upstream binding sites appeared to be relatively weak (40).

To evaluate the LTR mutants during infection, we generated virus from cells transfected with wild-type and mutant viral constructs. After infection of CEFs with equivalent reverse transcriptase units of virus, we quantified viral gene expression both by a reverse transcriptase assay of virus in the medium and by an RNase protection assay of intracellular viral RNA (Table 1). The results of these assays showed that mutation of individual LTR binding sites 1 and 2 led to approximately 50% of the wild-type level of virus, whereas the double mutation of sites 1 and 2 led to 30% of

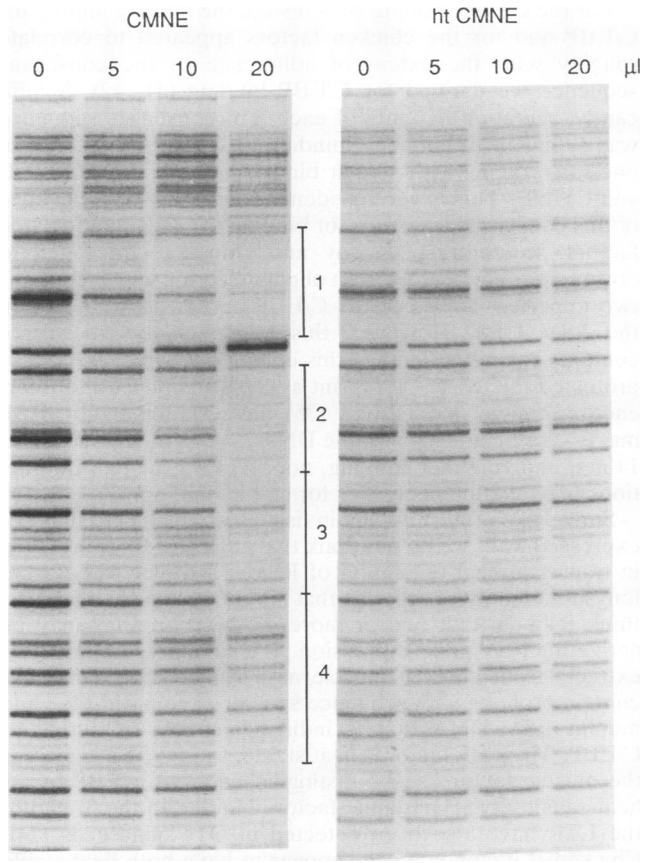

FIG. 6. CMNE footprinting of the Pr-C LTR C/EBP binding domains. Volumes of CMNE used in the footprint reactions are indicated at the top of the gel. The heat-treated (ht) CMNE footprint used nuclear extract samples which were first heated to 68°C for 10 min and subsequently clarified in a microcentrifuge for 5 min. This reduced the total protein concentration by about 10-fold. In experiments with extracts which have heat-stable binding factors, there is generally no diminution of footprint intensity (7, 40). In the middle, shown with brackets, are the positions of C/EBP binding sites 1, 2, 3, and 4 in the LTR.

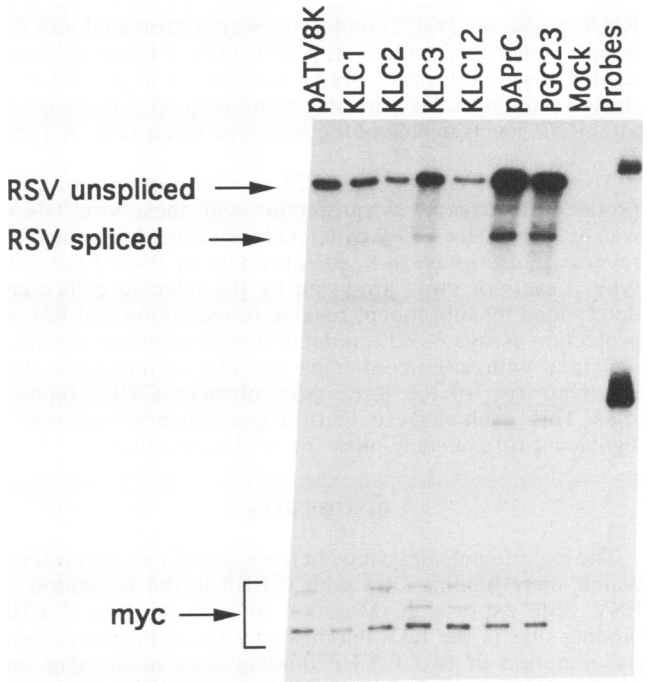

FIG. 7. Expression of viral RNA after transient transfection of mutants into CEFs. Both LTR and *gag* enhancer mutants were inserted into viral constructs and transfected into CEFs. The LTR mutants (KLC series) were all in the permuted Pr-C construct, pATV8K (9), while the *gag* double mutant was in the nonpermuted Pr-C construct, pAPrC (33). RNA levels were determined by RNase protection assays, described in the text. A pMyc23 minigene construct (32) was cotransfected as a control, and its expression was assayed simultaneously with that of the viral sequences.

TABLE 1. Levels of gene expression after mutation of the C/EBP binding sites in RSV DNA

Construct	Avg expression level (% of wild-type level) with assay:		
	Transient transfection-RNase protection	Viral infection	
		RNase protection	Reverse transcriptase
Wild type	100	100	100
KLC1	79	58	59
KLC2	62	46	50
KLC3	90	57	88
KLC12	42	31	29
PGC23	46	ND[a]	37

[a] ND, not determined.

wild-type virus production. As was seen in the transient transfection assays, mutation of site 3 had a smaller effect.

The effects of mutating the C/EBP binding sites within the *gag* enhancer were first assayed with a CAT reporter construct. In this plasmid, the *gag* enhancer lies downstream of the simian virus 40 promoter in a position and orientation similar to those of the intact virus (7). The results of these experiments showed that the CAT activity of the PGC23 enhancer, with two mutant C/EBP binding sites, was reduced to approximately 43% of that with the wild-type enhancer (data not shown). By comparison, the parental pSV1cat construct lacking an enhancer (16) yielded an average of 6% CAT activity.

To assay the effect of *gag* enhancer mutations on viral RNA levels, the PGC23 mutations were introduced into the nonpermuted viral construct, pAPrC (33). A transient transfection assay with these DNAs, carried out in parallel with those involving LTR mutants, demonstrated a decrease in viral RNA levels to 46% of the wild-type levels (Fig. 7; Table 1).

To study the effects of PGC23 on viral replication, virus produced 11 days after transfection with these viral DNAs was used to infect CEFs with levels of virus (determined by reverse transcriptase assays) equivalent to that of the wild type. Levels of virus produced by the infected cells were determined by subsequent reverse transcription and RNase protection assays. A 63% reduction in virus production was observed with virus containing the PGC23 mutations that inactivate two of the three *gag* enhancer C/EBP binding sites. This result suggests that the *gag* enhancer may play a significant role in some phase of viral replication.

DISCUSSION

The experiments described here suggest a role for proteins which share binding sites with C/EBP in the regulation of RSV gene expression. Mutation of each of three C/EBP binding sites in the LTR diminished viral replication, as did the mutation of two C/EBP binding sites in the *gag* enhancer. It is interesting that mutation of the *gag* enhancer at two C/EBP binding sites appeared to affect viral replication nearly as severely as did mutation of the LTR enhancer at sites 1 and 2 together. This is the first demonstration of functional significance for the *gag* enhancer element in assays using complete viral genomes. However, its mechanism of action is not yet clear. Since the *gag* enhancer mutations are within transcribed RNA, it is possible that they could affect RNA levels by decreasing the RNA stability. The binding of a known transcription factor, C/EBP, to the *gag* enhancer suggests that it may have a transcriptional role; however, further work will be necessary to ascertain whether the *gag* enhancer is acting at the transcriptional level. If so, it seems likely that mutation of multiple C/EBP sites in both the LTR and the *gag* enhancers would have even greater effects on viral RNA levels.

Mutation of 2 nts in each of the two most upstream C/EBP binding sites in the RSV LTR resulted in a 70% reduction in virus production in this study. Similarly, deletion of the 5′ end of the RSV LTR from −229 to −190 eliminates most viral enhancer activity (17). Laimins et al. (26), who carried out 5′ deletion analysis of the RSV LTR, observed a decrease in CAT activity of 95% when sequences including C/EBP binding sites 1 and 2 are removed. However, their 5′ deletions also removed an enhancer domain just upstream of the 3′ LTR that is present in Pr strains of RSV. Interestingly, we have observed C/EBP binding sites in this region of the Pr genome but not in SR RSV. It is possible that these binding sites may define the enhancer A domain described by Laimins et al. (26). Thus, multiple copies of an intact C/EBP consensus sequence seem to play an important role in viral replication.

For the C/EBP binding sites tested, the relative affinity for C/EBP and for the chicken factors appeared to correlate roughly with the extent of adherence to the consensus sequence we derived for C/EBP binding (41, 42). Significantly, mutation of 2 nts of each 8-nt consensus sequence was sufficient to eliminate binding of C/EBP, as well as of nuclear proteins with similar binding specificity in CEFNE and CMNE. This is good evidence to support the relevance of this consensus sequence for binding of C/EBP and related factors. In contrast, Kenny and Guntaka (24) failed to observe protein binding to an oligonucleotide which contains two imperfect copies of the C/EBP consensus sequence in the RSV LTR. However, this oligonucleotide does not contain sequences flanking this consensus, which were also protected in DNase I footprint assays (this paper and reference 41). Nye and Graves (35) have shown that C/EBP makes close contacts with the DNA major groove over 12 to 14 nts, implying that flanking, nonspecific base pair interactions may also be necessary for stable binding.

Since RSV replicates well in tissues in which C/EBP is not expressed well, we do not think that C/EBP itself is involved in transcriptional regulation of RSV. However, C/EBP belongs to a family of proteins that share binding sites, and we think it likely that one or more of these proteins may be activating RSV gene expression. We have prepared nuclear extracts from chicken muscle and from CEFs, and these contain proteins with sequence specificity for wild-type and mutant RSV DNA that is indistinguishable from that of C/EBP. The CEF factor is heat stable, as is C/EBP, whereas the muscle factor can be distinguished from C/EBP by its heat sensitivity. Heat-labile factors binding to the 5′ end of the LTR have also been detected in QT6 quail cells (24). Chicken B lymphoma cells appear to have both heat-stable and heat-sensitive factors that bind to the enhancer sequences near the 5′ end of the LTR (5, 39).

Interestingly, Ig/EBP-1 has also recently been shown to bind to C/EBP sites 1 and 2 in the RSV LTR (38). The sequence of Ig/EBP-1 is highly homologous to that of C/EBP in its carboxy terminus, which contains the DNA binding and leucine zipper regions, and the two proteins share DNA sequence specificity. It is heat stable and is found in roughly equivalent amounts in all tissues examined but is notably

lowest in muscle tissue. Thus, Ig/EBP-1 is a candidate for the binding factor observed in CEF cells.

NF-IL6, a transcriptional activator of interleukin 6 (IL-6), is also related to C/EBP in its carboxy-terminal amino acid sequence, and its DNA binding specificity is indistinguishable from that of C/EBP (1). NF-IL6 induction has been shown to correlate quantitatively, at the RNA level, with IL-6 expression (1). IL-6 is a cytokine present at low levels in a wide variety of cell types and induced in response to a diverse set of environmental insults, including viral infection (37). Surgical trauma has also been shown to induce high levels of IL-6 (37) and to promote tumor formation in chicken embryos infected with RSV (13). Thus, it is possible that RSV may utilize this transcriptional activator; however, NF-IL6 induction in response to RSV infection of fibroblasts has not been tested directly. If RSV is inducing this or a similar transcriptional activator upon infection, differences in methods of assaying the transcriptional activity (transfection versus infection) may be important.

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