Cis- and *Trans*-Acting Transcriptional Regulation of Visna Virus

Abstract. *Visna virus is a pathogenic lentivirus of sheep that is related to human T-cell lymphotropic virus type III (HTLV-III), the probable etiologic agent of the acquired immune deficiency syndrome (AIDS). The transcriptional activity of visna virus promoter and enhancer sequences was studied by means of an assay based on the transient expression of the bacterial gene chloramphenicol acetyltransferase (CAT). The results suggest that the high level of expression of visna virus is due in part to* cis*-acting enhancer sequences that give the viral promoter a high level of transcriptional activity. In addition, the rate of transcription from the visna virus promoter situated in a plasmid expressing the CAT gene was much greater in infected than uninfected cells. This phenomenon of* trans*-acting transcriptional activation may involve either virally or cellularly encoded factors.*

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The lentiviruses are a group of nononcogenic retroviruses that produce a variety of chronic progressive diseases with unusually long incubation periods. Visna virus, the prototype of this group of so-called "slow" viruses, causes chronic pneumonitis and a progressive demyelinating disease in sheep months to years after the initial infection (*1*). Interest in visna virus has increased considerably with the discovery by Gonda *et al.* (*2*) that the virus is morphologically similar to and shares sequence homology with human T-cell lymphotropic virus type III (HTLV-III), the presumptive etiologic agent of the acquired immune deficiency syndrome (AIDS) (*3*).

Visna virus replicates to high titers in tissue culture, eventually killing its host cell, a feature that distinguishes it from most other retroviruses. This high level of expression suggests that the visna virus promoter sequence has a high transcriptional rate in infected cells. Regulation of such transcription may depend on the promoter containing strong enhancer sequences—segments of DNA that increase the transcriptional rate of proximal promoters in a manner relatively independent of position and orientation (*4*). In addition, regulation may be achieved through the production of *trans*-acting transcriptional activators (*5–8*). Studies on HTLV-I, a virus associated with adult T-cell leukemia (*5*), HTLV-II, which is associated with hairy cell leukemia (*5*), HTLV-III (*6*), and bovine leukemia virus (BLV), which is associated with B-cell leukemia of cattle (*7, 8*), indicate that transcription from the long terminal repeats (LTR's) of all of these viruses is much greater in infected than uninfected cells. Recently, transcription from the Rous sarcoma virus (RSV) promoter was shown to be increased by a product of the *gag* gene, providing further evidence for *trans*-acting factors playing a role in the regulation of retroviral transcription (*9*).

Here we report the nucleotide sequence of the U3 region of visna virus, and present evidence that both *cis*-acting enhancer sequences and *trans*-acting transcriptional activating factors contribute to the high rate of visna virus transcription in infected cells.

Promoter and enhancer sequences that govern the initiation and rate of retroviral RNA transcription are located within the U3 region of the retroviral LTR (*4, 10*) (Fig. 1, top). The nucleotide sequence of this region of visna virus (Fig. 1, bottom) shares little homology with other retroviruses, including BLV, HTLV-I, HTLV-II, or HTLV-III, or other retroviruses isolated from AIDS patients (*11*). Eukaryotic and viral promoters often contain the sequence CCAAT 70 to 90 base pairs (bp) 5′ to the cap site (the start site for RNA transcription) (*12*), and the sequence $TATA^{A}_{T}A^{T}_{A}$ (the TATA box) 20 to 30 bp 5′ to the cap site (*13*). Sequences similar to the CCAAT consensus sequence appear in the visna LTR beginning with bases 352 (CAAAT) and 364 (CAAGT). A well-defined TATA box (TATATAA) begins with base 438. On the basis of the position of these promoter elements we place the cap site on a guanine residue somewhere between bases 460 to 480. There is no polyadenylation signal in this sequence.

Enhancer sequences of retroviruses often appear as short (40 to 80 bp) tandemly repeated sequences located 100 to 250 bp upstream from the RNA cap site. The U3 region of visna virus contains a pair of 43-bp perfect tandem repeats spanning the region approximately 100 to 180 bp upstream from the putative cap site. These repeats do not contain a good example of the "core" sequence $GTGG^{AAA}_{TTT}G$ which is found in many viral and eukaryotic enhancers (*4*), but the juxtaposition of the tandem repeats with classical promoter elements strongly suggests that they function as enhancer sequences.

We tested the ability of visna virus sequences to function as a promoter by

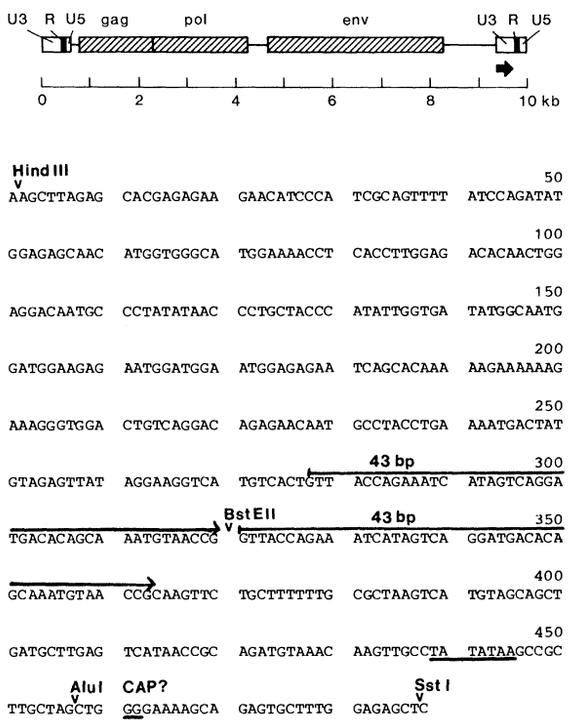

Fig. 1. (Top) Organization of the proviral form of visna virus DNA. Positions of the *gag*, *pol*, and *env* coding regions are approximate. The arrow indicates the position of the Hind III–Sst I fragment used for sequencing, and for construction of recombinant plasmids. The cloning of visna virus from unintegrated DNA has been described (*26*). (Bottom) Nucleotide sequence of the Hind III–Sst I DNA fragment spanning the U3 region of the visna virus 3′ LTR. This fragment was subcloned into bacteriophage M13 (*27*) and sequenced by the method of Sanger *et al.* (*28*). The 43-bp tandem repeats are indicated with arrows, and the TATA box and possible RNA transcription initiation sites are underlined. Restriction sites used for constructing recombinant plasmids are also labeled.

constructing plasmids in which portions of the U3 region of the virus containing either one (pVIS1CAT) or both (pVIS-2CAT) 43-bp tandem repeats, including the region containing the TATA box, were inserted 5′ to the bacterial gene chloramphenicol acetyltransferase (CAT) (Fig. 2) (*14, 15*). These plasmids were transfected into a variety of eukaryotic cells by the DEAE-dextran method (*16*) coupled with an osmotic shock (10 percent dimethyl sulfoxide) (*17, 18*). The CAT activity of cell extracts was measured by an enzyme assay 48 hours later (*19*). CAT activity is closely correlated with CAT-related messenger RNA at this time (*14, 15*), providing a sensitive way to quantitate the transcriptional rate of transfected plasmids.

The plasmids pSV2CAT and RSVCAT, containing the SV40 early region promoter and RSV promoters, respectively, 5′ to the CAT gene (*14, 15*), were included in the assays along with plasmids containing visna viral sequences for comparison (Table 1). The percentage conversion of ^{14}C-labeled chloramphenicol to its acetylated derivatives per hour of incubation was normalized to the percentage conversion by pRSVCAT. The results (Table 1) indicate that the visna promoter, with either one (pVIS1CAT) or both (pVIS2CAT) 43-bp tandem repeats, directed high and equivalent levels of transcriptional activity in a variety of cell types. In mouse L cells, the CAT activity directed by pVIS-1CAT and pVIS2CAT was comparable to that directed by pSV2CAT. The results were similar in H9 cells, a human T-lymphocyte cell line in which HTLV-III replicates to high titers. In sheep choroid plexus (SCP) cells and SV40-transformed sheep macrophages (both being cells in which visna virus replicates) the levels of CAT activity directed by pVIS-1CAT or pVIS2CAT were more than tenfold higher than pSV2CAT.

The presence of 43-bp tandem repeats upstream from classical promoter elements suggested that these repeats might

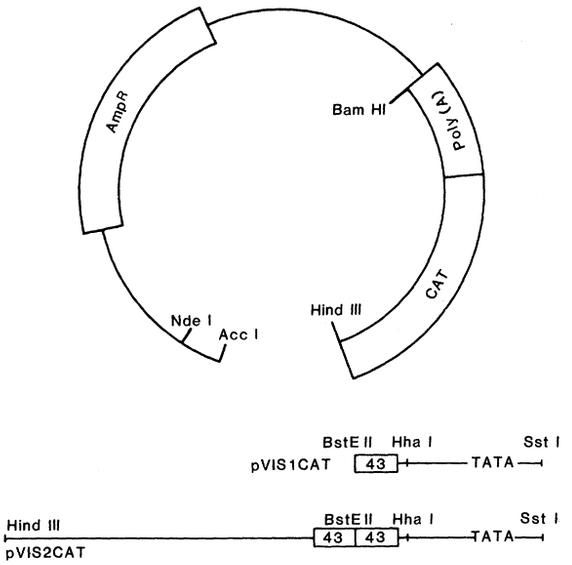

Fig. 2. Construction of plasmids that express the bacterial CAT gene under the transcriptional control of visna promoter sequences. pSV2CAT contains the CAT gene 3′ to the SV40 early region promoter (*14*). pVIS2CAT is derived from pSV2CAT by replacing the SV40 promoter and pBR322 sequences between the Acc I and Hind III sites with a 488-bp Hind III–Sst I fragment of visna DNA containing promoter sequences and both tandem repeats. The ends of both the vector and insert were made blunt ended by using Klenow enzyme and T4 DNA polymerase, and were joined so the Hind III end of the viral insert was ligated to the Acc I site of the plasmid; the Sst I end of the insert was ligated to the Hind III end of the insert. pVIS1CAT was derived from pVIS2CAT by cleaving with Nde I and Bst EII, deleting 50 bp of pBR322 sequence and 170 bp of 5′ visna virus DNA. The endonuclease-cut plasmid was treated with Klenow enzyme and religated. This plasmid contains 170 bp of visna DNA which includes one 43-bp repeat and the downstream viral promoter elements. All plasmids were gradient-purified prior to use in transfections.

Table 1. Transient expression of the CAT gene in uninfected and visna virus–infected cells. The results represent the average of a minimum of three separate transfections and are expressed as the ratio of the percentage conversion of chloramphenicol to its acetylated forms by a given plasmid, compared to the percentage conversion directed by pRSVCAT in cells transfected in parallel. Results were highly reproducible with variation between values less than 40 percent in most cases. For the methods used for transfections and CAT assays, see (*18, 19*).

Cell type	pVIS1CAT Visna/visna*	pVIS2CAT Visna/visna*	pSV1-3′CATs Visna/SV40*	pSV1-3′CATa Visna/SV40*	pSV1CAT SV40†	pSV2CAT SV40/SV40*	pRSVCAT RSV/RSV*
Mouse L cells	10.1	9.4	9.7	2.9	0.1	12.6	1.0
Human H9 cells	0.6					0.5	1.0
Goat synovial membrane (GSM)	2.6	2.8				1.3	1.0
Sheep alveolar macrophages							
Uninfected	11.2	12.0				0.5	1.0
Infected	40.0	33.9				1.0	1.0
SCP cells							
Uninfected	12.0	10.0	0.7	0.6	0.4	1.8	1.0
Infected	83.7	69.9	5.2	2.2	0.6	5.0	1.0

*Enhancer/promoter. †Promoter.

Table 2. Expression of the CAT gene directed by plasmids with an SV40 promoter and visna enhancer sequences. The L cells were transfected as described in the text with the plasmids described in Fig. 2. The results represent the average of three separate transfections and are expressed as the ratio of the percentage conversion of chloramphenicol to its acetylated forms by a given plasmid compared to the percentage conversion by pSV1CAT. The range of values observed for each plasmid is indicated in parentheses.

	pSV1-5′CATs Visna/SV40*	pSV1-5′CATa Visna/SV40*	pSV1-3′CATs Visna/SV40*	pSV1-3′CATa Visna/SV40*	pSV1CAT SV40†	pSV2CAT SV40/SV40*
CAT activity related to pSV1CAT	21.9	6.8	68.7	20.7	1.0	89.1
Range of values	(15.8–26.3)	(6.0–7.6)	(57.7–81.7)	(17.6–25.0)		(70.6–110.5)

*Enhancer/promoter. †Promoter.

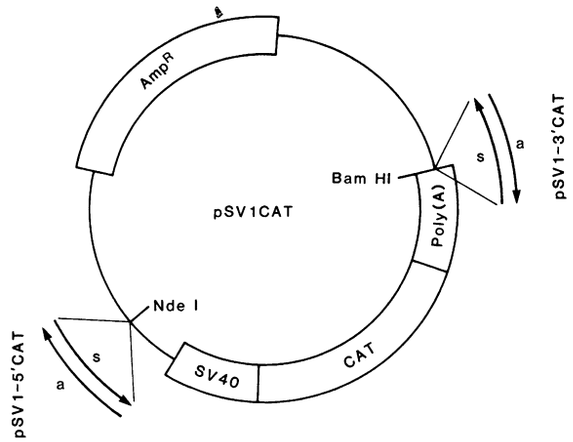

Fig. 3. Construction of plasmids for testing the ability of visna virus sequences to enhance transcription from the SV40 promoter. pSV1CAT has an SV40 promoter deleted of enhancer sequences and was derived from pSV2CAT by deleting sequences from Acc I to the distal Sph I site. A 457-bp Hind III to Alu I visna fragment containing both 43-bp tandem repeats (in addition to viral promoter sequences) was inserted into pSV1CAT at the Nde I site in either the sense (pSV1-5's) or antisense (pSV1-5'a) orientation; this fragment was also inserted into the Bam HI site in either the sense (pSV1-3's) or antisense (pSV1-3'a) orientation. Plasmids were constructed by treating both the vector and viral DNA insert with Klenow enzyme, followed by blunt end ligation as described (*29*). Plasmids were gradient-purified prior to use in transfections.

function as enhancer sequences. We next examined the ability of these sequences to enhance transcription from an SV40 promoter from which its own enhancer sequences had been deleted. Plasmids were constructed with the visna U3 region inserted both 3' and 5' to the SV40 promoter in both sense and antisense orientations (Fig. 3). These plasmids were transfected into mouse L cells, and CAT activity was measured 48 hours later. The results are expressed as the ratio of the percentage acetylation of ^{14}C-labeled chloramphenicol by a given plasmid divided by the percentage acetylation by pSV1CAT, a plasmid containing the "enhancer-less" SV40 promoter without any visna virus DNA insert (Table 2). These data show that the visna insert, which contains both 43-bp repeats, functions as a strong enhancer. As has been found in other viruses (*20*), the enhancer sequences showed an orientation preference, working three to four times better in the sense as opposed to the antisense orientation. The visna virus enhancer sequences are unusual in that they appear to function better when positioned downstream rather than 5' to the SV40 promoter.

The *trans*-acting transcriptional activation recently described in the human T-cell lymphotropic viruses, BLV, and Rous sarcoma virus (RSV) prompted us to look for a similar activation of the visna promoter in infected cells. We introduced the plasmids already described into SCP cells and alveolar macrophages 24 hours after they were infected with visna virus. In SCP cells, the CAT activity directed by pVIS1CAT (containing the visna promoter and one 43-bp repeat) was substantially higher in infected than uninfected cells (Table 1). In infected SCP cells, the ratio of the activity directed by pVIS1CAT to that directed by pRSVCAT was approximately sevenfold greater than the ratio in uninfected SCP cells. The amount of CAT activity in infected macrophages was roughly three times higher than that in uninfected cells. Results for SCP cells and macrophages are the average of three separate transfections. Variation among values was less than 40 percent in infected cells and less than 30 percent in uninfected cells. To ensure that these effects were not due to differences in DNA uptake between infected and uninfected cells, DNA dot blots were done to quantitate the amount of plasmid DNA in infected and uninfected cells 3, 24, and 48 hours after transfection. These dot blots confirmed that DNA uptake in infected and uninfected cells was equivalent, and also showed that the plasmids did not replicate in either group of cells.

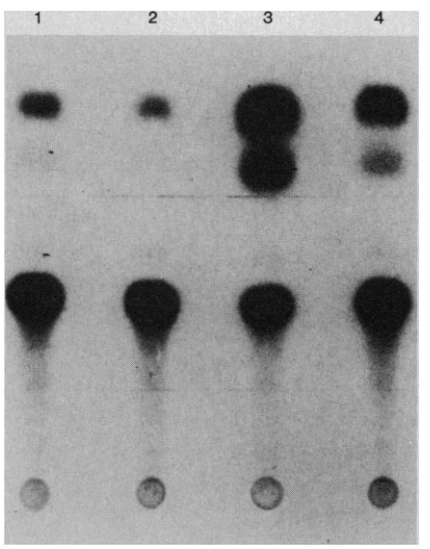

Fig. 4. A representative CAT assay on infected and uninfected SCP cells. SCP cells were either infected or "mock" infected with visna virus strain 1514 at a multiplicity of 0.1 infectious unit per cell. The cells were transfected 24 hours later, and collected for CAT assay 72 hours after infection. Infected and uninfected cell extracts contained equivalent amounts of protein and equal amounts of plasmid DNA as determined by DNA dot blots. Lane 1, pVIS-1CAT (uninfected); lane 2, pSV2CAT (uninfected); lane 3, pVIS1CAT (infected with visna virus); and lane 4, pSV2CAT (infected with visna virus).

The results of the CAT assays indicate that the visna virus promoter is not dependent on virally encoded or induced *trans*-acting factors for activity. The high transcriptional activity of the visna LTR can be explained in part by the presence of strong enhancer sequences, probably contained within the 43-bp tandem repeats of the viral promoter. That the visna LTR can direct high levels of CAT activity in both fibroblastic and lymphoid cells suggests that the visna enhancer sequences show relatively little tissue specificity and in this respect are similar to those of SV40.

In addition to increasing transcription from the visna promoter, cells infected with visna virus also activate transcription from the promoter of caprine arthritis-encephalitis virus (CAEV) (*21*), another macrophage-tropic lentivirus, to the same extent as the visna promoter. Since the sequences of the U3 regions of the two viruses are somewhat divergent, but their tandem repeats are closely homologous, it seems likely that the *trans*-acting factor interacts with these putative enhancer sequences. In SCP cells infected with visna, transcription from the SV40 promoter was consistently increased (typically two- to threefold), whereas transcription from pRSVCAT was unaffected. This indicates that there is some latitude in the ability of the *trans*-acting factor to activate transcription from promoters other than the viral LTR. The result is similar to that for the RSV encoded *trans*-acting factor, which activates transcription of the rat preproinsulin II gene in addition to the RSV LTR (*9*).

The magnitude of transcriptional activation by visna virus infection is not as large as observed for HTLV-I, HTLV-II, HTLV-III, and BLV (*5–8*). We believe that the degree of activation observed in visna-infected SCP cells is limited in part by the cytopathic effects of virus replication. The absence of the R region in the visna viral DNA used to transcribe the CAT gene in our experiments may also have limited the extent of *trans*-activation we observed. Work by Derse *et al.* has shown that *trans*-acting transcriptional activation of the BLV promoter is reduced by almost 90 percent when most of the R region of the viral LTR is deleted (*8*).

The phenomenon of *trans*-acting transcriptional activation occurs in several DNA viruses. Transcription from the adenovirus early transcriptional units is increased by the Ela gene product (*22*), and similar transcriptional enhancement occurs with the immediate early gene of herpes simplex virus type I (*23*). There is also evidence that the SV40 T antigen activates transcription of late region genes of SV40 (*24*). The recent discovery of *trans*-acting transcriptional activation in several retroviral systems has prompted speculation that these viruses, like some DNA viruses, code for their own *trans*-acting enhancer proteins (*5–7*). In support of this theory, spliced RNA transcripts and protein products encoded by additional open reading frames have been detected in some infected cells (*25*). In the case of RSV, a region of the viral genome has been identified which activates transcription in *trans* from a plasmid containing the viral LTR (*9*). Our experiments suggest that cells infected with visna virus also produce a *trans*-acting factor. However, we have not identified a viral gene product responsible for transcriptional activation of the visna LTR, and we cannot exclude the possibility that the *trans*-acting transcriptional activator is a cellular protein that is induced by viral infection.

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18. Nearly confluent monolayers of L cells, SCP cells (*30*), sheep alveolar macrophages (*31*), and goat synovial membrane cells (*32*) grown in 35-mm dishes were transfected by using the DEAE-dextran technique coupled with a dimethyl sulfoxide shock (*16, 17*). The SCP cells or sheep macrophages were infected by adding visna virus (strain 1514) 24 hours prior to transfection at multiplicities of 0.1 (SCP) or 1.0 (macrophages). Cells to be transfected were washed with serum-free medium and then DNA was added (3 μg/ml in 1 ml of serum-free medium containing DEAE-dextran, 200 μg/ml). After 4 hours at 37°C the cells were treated with 1 ml of Hepes buffered saline, *p*H 7.1 (*31*) containing 10 percent dimethyl sulfoxide for 2 minutes at room temperature. The cells were washed once with phosphate-buffered saline (PBS), then modified Eagle's medium containing 10 percent fetal bovine serum (Dulbecco's modified Eagle's medium containing 2 percent lamb serum was used for the sheep macrophages) was added and the cells were incubated for 48 hours at 37°C. The H-9 cells were transfected as described elsewhere (*6*).
19. Cells grown in monolayers were harvested for CAT assay by trypsinzation. The cells were washed twice with cold PBS (*p*H 7.4), then lysed by freeze-thawing three times in 60 μl of 250 m*M* tris-HCl (*p*H 7.8). Cell debris was pelleted by centrifugation, and 30 μl of the cellular supernatant was assayed for CAT activity as described (*14, 15*). The percentage acetylation of ^{14}C-labeled chloramphenicol (New England Nuclear) after 1 or 2 hours was measured by separating the acetylated and unacetylated forms by thin-layer chromatography, then counting spots cut from the plate by liquid scintillation. The results are expressed as the percentage conversion of ^{14}C-labeled chloramphenicol to its acetylated derivatives per hour normalized to the percentage conversion by either pRSVCAT (Table 1) or pSV1CAT (Table 2).
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Carbyne Forms of Carbon: Evidence for Their Existence

The report by Smith and Buseck (*1*) casts doubt on the existence of carbyne forms of carbon. However, the existence of this carbon form was clearly established in a number of ways many years ago. In a laser-Raman study, Nakamizo *et al.* showed that a solid form of carbon containing triple bonds could be prepared (*2*). This, by definition, is a carbyne form of carbon. Russian investigators produced a large body of data on the preparation and properties of carbynes. Most of their work is summarized in a review article by Sladkov (*3*), which gives data on the electronic structure, infrared and Raman spectra, and electrophysical, thermo-physical, and chemical properties of carbynes. It is difficult to understand how so many data can have been obtained by a dozen or more investigators on something that does not exist.

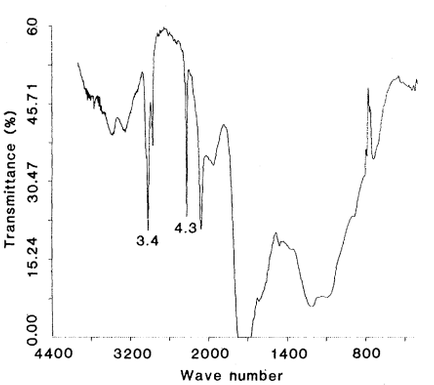

Fig. 1. Infrared spectrum of a carbyne film (*4*).

Recently I have been engaged in a study of carbyne films prepared by quenching carbon gas on a polished metal substrate. Absorption data were obtained on these films from 0.2 to 25 μm. An infrared spectrum of a carbyne film is shown in Fig. 1 (*4*). The spectrum shows features different from those found in the spectra of graphite and diamond. In particular, they show the –C≡C– resonance at ~4.3 μm and a feature that occurs at ~3.4 μm. This is close to the value of 3.1 μm predicted by Webster on theoretical grounds (*5*). In addition, these films gave ion-probe spectra showing carbon molecule ions (no silicate fragments) and electron diffraction patterns that correspond to those associated with carbynes (*6, 7*).

Smith and Buseck did not make use of all the diffraction data available. Had they done this, they would not have confused chaoite with other minerals. Since they used nontronite and quartz extensively in their report, I will use these minerals to illustrate this point. It is rather surprising that Smith and Buseck did not include the strongest reflection for both nontronite and α quartz in their table 1 (*1*). If the strongest reflection for nontronite (d_{001} = 14.7 Å) had been included, it would have been immediately obvious that chaoite (or any other carbyne) could not be confused with