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Only some members of a gene family in *Trypanosoma cruzi* encode proteins that express both *trans*-sialidase and neuraminidase activities

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Trypomastigotes, the blood stage form of the human parasite *Trypanosoma cruzi*, contain an enzyme on their surface, *trans*-sialidase, which catalyses the transfer of sialic acid from host glycoconjugates to acceptors on its own cell surface. At least a subset of the sialic acid-bearing acceptor molecules are involved in parasite invasion of host cells, an essential step in the life cycle of the parasite. Another trypomastigote surface enzyme that affects host cell invasion is neuraminidase and recent evidence suggests that both *trans*-sialidase and neuraminidase activities may be expressed by the same proteins on the parasite surface. We describe here the isolation and expression of several members of a *trans*-sialidase–neuraminidase gene family from *T.cruzi*. One of the isolated genes does indeed encode a protein with both *trans*-sialidase and neuraminidase activities, while other members of the gene family encode closely related proteins that express neither enzymatic activity. Chimeric protein constructs combining different portions of active and inactive genes identified a region of the gene necessary for enzymatic activity. Sequence analysis of this portion of the gene revealed a limited number of amino acid differences between the predicted active and inactive gene products.

Key words: gene/neuraminidase/*trans*-sialidase/trypomastigote

Introduction

Trypanosoma cruzi is the obligatory intracellular protozoan parasite that causes Chagas' disease. In order to replicate, the blood-borne trypomastigote form of the parasite must invade host cells. The molecular mechanism of this invasion has not been established, but much evidence indicates that the recognition of target cells is receptor mediated (Meirelles *et al.*, 1983; Boschetti *et al.*, 1987). Among the surface antigens of *T.cruzi* trypomastigotes are molecules that carry the stage specific, sialylated epitope, Ssp-3 (Andrews *et al.*, 1987; Schenkman *et al.*, 1991a). Several lines of evidence indicate that the Ssp-3-bearing molecules are involved in attachment to (Schenkman *et al.*, 1991a) and penetration of host cells (Schenkman *et al.*, 1991b).

T.cruzi does not synthesize sialic acid, (Schauer *et al.*, 1983) and trypomastigotes just released from host cells do not express Ssp-3 epitopes (Frevert *et al.*, 1992). Nevertheless, sialic acid is rapidly acquired by the emerging parasites by means of a surface-associated *trans*-sialidase. This enzyme removes sialic acid from host derived glycoconjugates and attaches it via alpha(2–3) linkage to acceptors on the trypomastigote cell surface (Schenkman *et al.*, 1992), thereby generating the Ssp-3 epitope.

Others (Pereira, 1983; Cavallesco and Pereira, 1988; Prioli *et al.*, 1990; Pereira *et al.*, 1991) have described a neuraminidase on the trypomastigote surface and reported that trypomastigote invasion of host cells is enhanced when this neuraminidase is inactivated by antibodies or by serum lipoproteins (Cavallesco and Pereira, 1988; Prioli *et al.*, 1990). The relationship of these two seemingly counteracting cell surface enzyme activities has recently been examined. The results of these studies strongly suggest that the same protein can catalyse both the release and transfer of sialic acid and that these two activities represent coupled steps of the same reaction mechanism. (Parodi et al., 1992; Schenkman *et al.*, 1992).

We have attempted to demonstrate directly that a single *T.cruzi* protein functions as a *trans*-sialidase and a neuraminidase. For this purpose we have cloned genes encoding *trans*-sialidase–neuraminidase and analysed their products for the two enzyme activities.

Results

DNA cloning of trypomastigote specific trans-sialidase genes

In a previous paper it was shown that monoclonal antibody TCN-2, (mAb TCN-2), directed against the C-terminal repeat region of *T.cruzi* trypomastigote-specific neuraminidase (Pereira *et al.*, 1991), immunoprecipitates both neuraminidase and *trans*-sialidase activities from trypomastigote extracts (Schenkman *et al.*, 1992), suggesting that *trans*-sialidase also contains the sequence of the neuraminidase repeat. Accordingly, a *T.cruzi* genomic library was screened with oligonucleotide NA-C1, which contains codons for 11 of the 12 amino acids in the C-terminal repeat unit of *T.cruzi* neuraminidase (Pereira *et al.*, 1991). 175 000 lambda clones were screened, yielding 21 positives. Replica filters were screened with another oligo, NA-N2, whose encoded amino acid sequence represents one of the consensus repeats SXDXGXTW found in the N-terminal region of *T.cruzi* neuraminidase (Pereira *et al.*, 1991) and *T.cruzi* SAPA antigens, (Pollevick *et al.*, 1991), and whose sequence is conserved in bacterial and viral neuraminidases (Roggentin *et al.*, 1989). Twenty-five plaques hybridized to NA-N2 and 13 were positive for both NA-C1 and NA-N2. After induction with IPTG, the oligo-positive phage were analysed for protein expression with mAb 39, which recognizes trypomastigote *trans*-sialidase (Schenkman *et al.*, 1992). Insert fragments from nine

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antibody-positive phage were converted into plasmid form for further analysis.

Figure 1 shows the alignment of the inserts from eight independent clones with the gene encoding neuraminidase described by Pereira *et al.* (1991). The structural homology of the clones to the neuraminidase gene is readily apparent, with the conservation of oligo-hybridizing sequences at the expected relative positions as compared with the predicted open reading frame (ORF) of neuraminidase. At this level of characterization some of the inserts appeared to contain full-length genes and all clones expressed some form of protein recognized by mAb 39 in Western blots, although these products were smaller than parasite-derived enzyme preparations (data not shown). None of the genes in this form expressed detectable *trans*-sialidase activity.

To determine if in fact the complete coding region was present in any of the clones oligonucleotides NA-C3 and NA-N4 were used (Figure 1). NA-C3, 5′-GCAGTTTTC*TGA*-TGTAGTGAGAGA, is a 24mer of neuraminidase sequence that overlaps the termination codon and NA-N4, 5′-ACGGC-TGTTCTTGATGGCAATTTG, is contained within the N-terminal region of the neuraminidase ORF. Four plasmids containing inserts consistent with the presence of a single gene hybridized to both oligos, indicating that they probably contained the full coding region (compared with neuraminidase). In an attempt to increase levels of expression, a major portion of the 5′ untranslated region of three of these clones, 121, 151 and 154, was removed by *Sac*II digestion and religation. *Escherichia coli* XL-1 Blue cells were transformed with these shortened plasmids and protein synthesis was induced with IPTG. All three genes encoded protein products that migrated as a cluster of high molecular weight bands recognized by mAb 39 (Figure 2A). The products of clones 121 and 151 appeared to be slightly smaller than those of clone 154. Lysates of transformants containing clone 154 expressed both *trans*-sialidase and neuraminidase activities, while clone 121 and 151 transformants failed to express either activity, (Figure 2B and C).

Construction and enzyme activities of chimeric proteins

Since there appeared to be functional differences between clones 121 and 151 compared with clone 154, a more refined restriction map of their inserts was generated (Figure 3). The three clones, as well as the neuraminidase gene (Pereira *et al.*, 1991), contain conserved restriction sites, which are indicated below each linear map. Clones 121, 151 and 154 contain *Xho*I, *Pml*I and *Pst*I sites not present in neuraminidase. As shown, the enzymatically active clone 154 differs from the inactive clones 121 and 151 by the presence of a larger C-terminal region, most probably indicating additional repeat units and by the presence of an additional *Eco*RI site within the expected coding region (compared with neuraminidase). We also found that clones 121 and 151 hybridize more efficiently than clone 154 with oligo NA-N2, whose sequence is derived from the second consensus SXDXGXTW box of *T.cruzi* neuraminidase: the NA-N2 probe is washed out at 65°C, 1 × SSC, from clone 154 but not from clones 121 or 151, (Figure 1).

We next examined the relevance of these structural differences to enzyme activities by constructing hybrid genes. To determine the effect of variations in the C-terminal region containing the repeats, recombinant plasmids were generated using the *Mlu*I site located just 5′ to the region of neuraminidase with homology to type III modules of

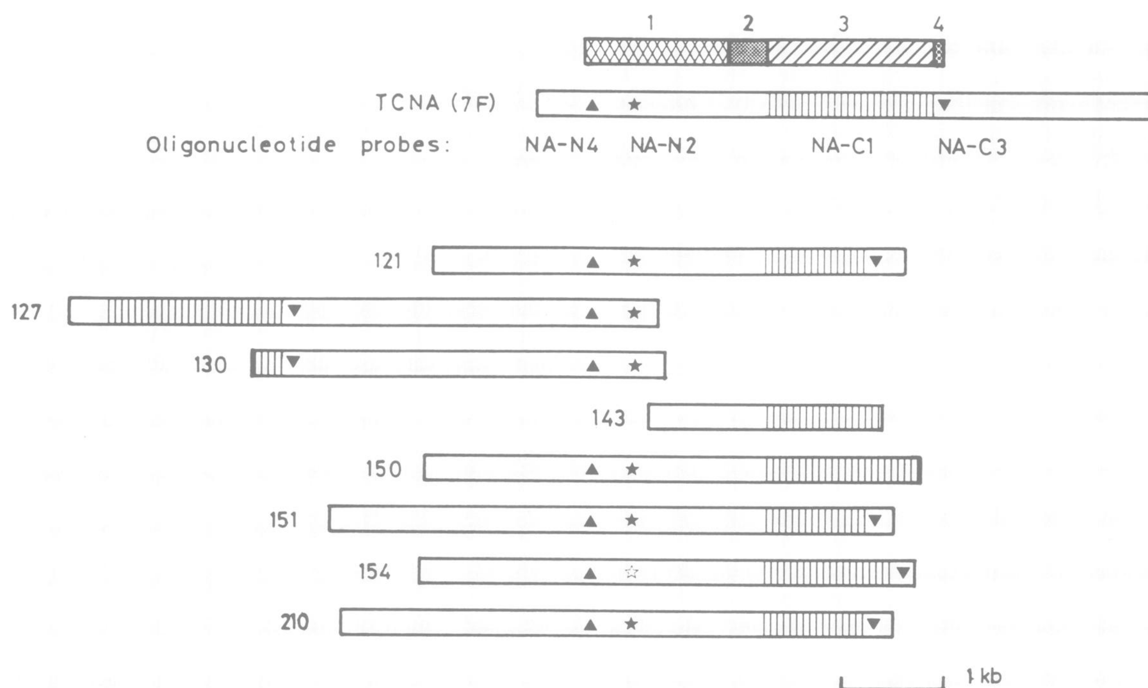

Fig. 1. Schematic representation of the inserts from individual clones. A reported neuraminidase gene, TCNA(7F), is shown with the predicted monomeric protein structure model above it (Pereira *et al.*, 1991). The protein was described as containing four domains: 1, an N-terminal cysteine-rich region containing four copies of the SXDXGXTW consensus repeat unit; 2, a fibronectin type III module; 3, a C-terminal long tandem repeat region; 4, a hydrophobic segment. Below are indicated the sizes of the inserts of eight clones isolated via hybridization to specific oligonucleotides. Hybridization of the listed oligonucleotide probes to the clones is indicated with the various symbols shown on the TCNA(7A) map. The open star on clone 154 represents the loss of hybridization of oligo NA-N2 at high stringency.

fibronectin. Construct 154–*Mlu*I–121, where the first number represents the clonal source of the 5′ portion of the gene followed by the site at which joining occurred then the 3′ source of the gene, when transfected into *E.coli*, expressed *trans*-sialidase and neuraminidase activities at levels similar to 154 itself, while 121–*Mlu*I–154 and 151–*Mlu*I–154 expressed neither activity, (Figure 4). Therefore the differences in enzyme activities in the original 121, 151 and 154 clones are probably not due to variations in the C-terminal repeat-containing region.

Further constructs were then made in order to map the site associated with enzyme activity within the N-terminal half of the protein. The results of the experiments summarized in Figure 4 indicate that the difference between the active and inactive genes resides within a fragment defined by the *Xho*I site at the 5′ end and the *Eco*NI site at the 3′ end. The enzymatically active recombinant constructs either expressed both *trans*-sialidase and neuraminidase or expressed neither, i.e. the restriction sites used to generate the recombinants did not separate the two enzymatic activites in the protein products.

Expression of trans-sialidase genes in COS-1 cells

Eukaryotic proteins expressed in *E.coli* often fail to express expected activities due to their instability in bacteria, improper folding or lack of required secondary modifications. To rule out such reasons for the inactivity of clones 121 and 151, all three genes were cloned into the eukaryotic expression vector pCDM-8 (Aruffo and Seed, 1987) and then transfected into COS-1 cells. In each case the entire gene, from the 5′ *Sac*II site, was inserted into the expression vector and the orientation confirmed by restriction

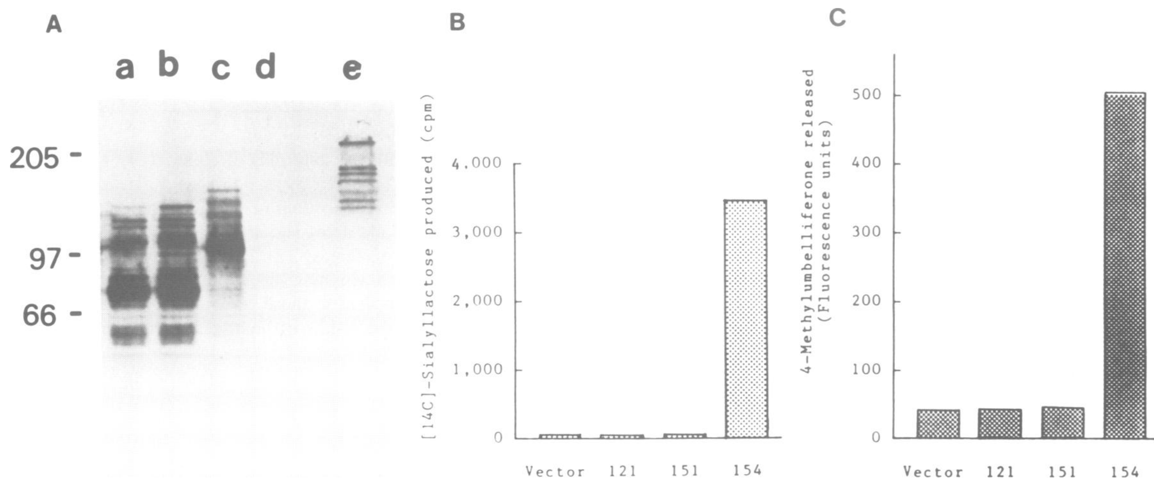

Fig. 2. Immunoblot and expression analysis of *trans*-sialidase gene products from *E.coli*. **A.** Extracts of *E.coli* expressing pBluescript clones of *trans*-sialidase genes were fractionated by 8% SDS–PAGE, transferred to a nitrocellulose filter and probed for *trans*-sisalidase protein with mAb 39. Lane a, clone 121; b, clone 151; c, clone 154; d, vector without insert; e, culture supernatant of T.cruzi trypomastigotes. **B.** Lysates of bacteria expressing clones 121, 151, 154 and vector alone were assayed for *trans*-sialidase activity by incubating aliquots corresponding to 50 μl of culture in a 30 min *trans*-sialidase reaction. **C.** Aliquots of the same lysates assayed for *trans*-sialidase were also assayed for neuraminidase activity. Amounts of lysate corresponding to 1.2 ml of culture were incubated for 4 h in the neuraminidase reaction.

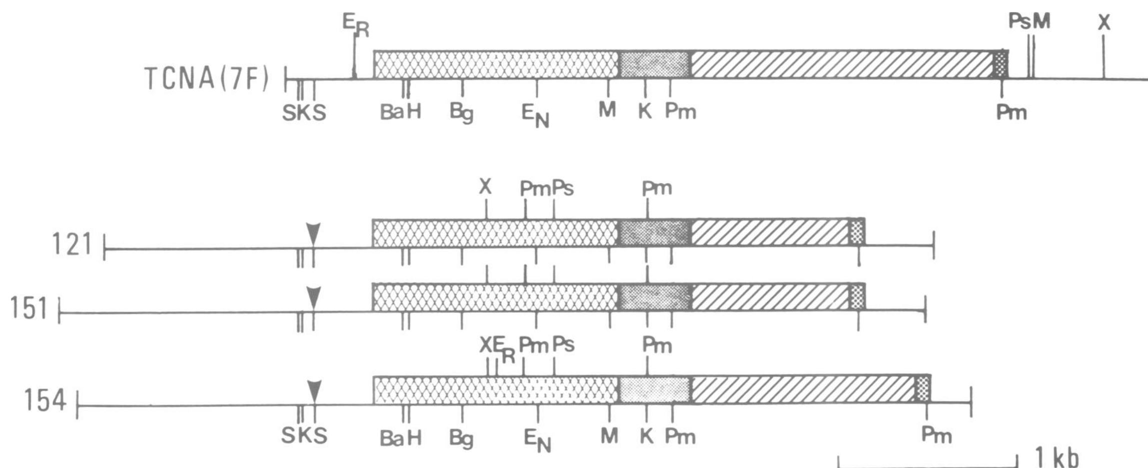

Fig. 3. Comparison of features of *trans*-sialidase gene family members. Shown here are more detailed restriction maps of clones 121, 151 and 154 inserts and the neuramindase clone TCNA(7F) of Pereira *et al.* (1991). Boxed areas indicate expected coding regions. Restriction sites conserved in all three clones and TCNA(7F) are shown below each map and sites differing amongst the four genes are indicated above each map. Restriction sites are abbreviated as follows: Ba, *Bam*HI; Bg, *Bgl*II; E_N, *Eco*NI; E_R, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; Pm, *Pml*I; Ps, *Pst*I; S, *Sac*II; X, *Xho*I. The conserved *Sac*II sites, used to remove 5′ untranslated region from each clone, are indicated by arrows.

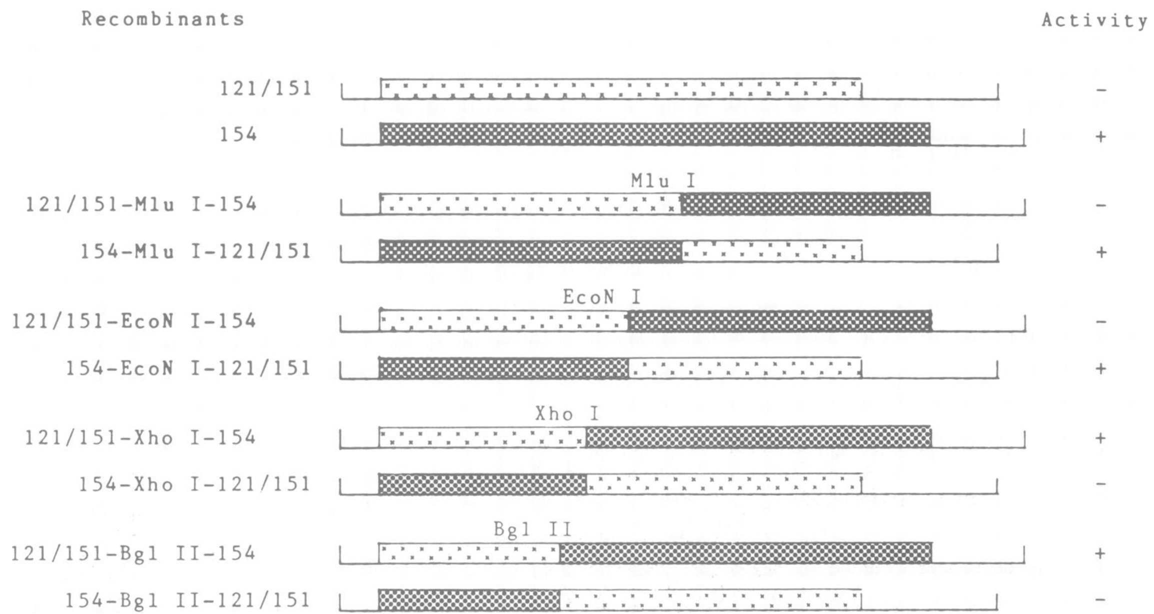

Fig. 4. Schematic diagram of chimeric proteins and their enzyme activities. *Sac*II-digested and religated plasmids of clones 121, 151 and 154 were used as the parental expression forms. Recombinant plasmids were prepared using the uniquely conserved restriction sites indicated and then expressed in *E.coli* XL-1 Blue. The integrity of each construct was verified by demonstrating the regeneration of the restriction site utilized as well as by examination of protein expression on Western blots with mAb 39, (data not shown). Since the maps and activities of clones 121 and 151 were identical, the independent constructs made with fragments from these genes are here considered together as 121/151. Bacterial lysates from at least four individual colonies for each construct were assayed for both *trans*-sialidase and neuraminidase activities, the results of which are indicated to the right of each construct map with the symbols + or −. A plus indicates enzyme levels were equivalent to those obtained from the full-length 154 clone positive control and minus indicates that no activity was detected. Activity here represents the results of both neuraminidase and *trans*-sialidase assays.

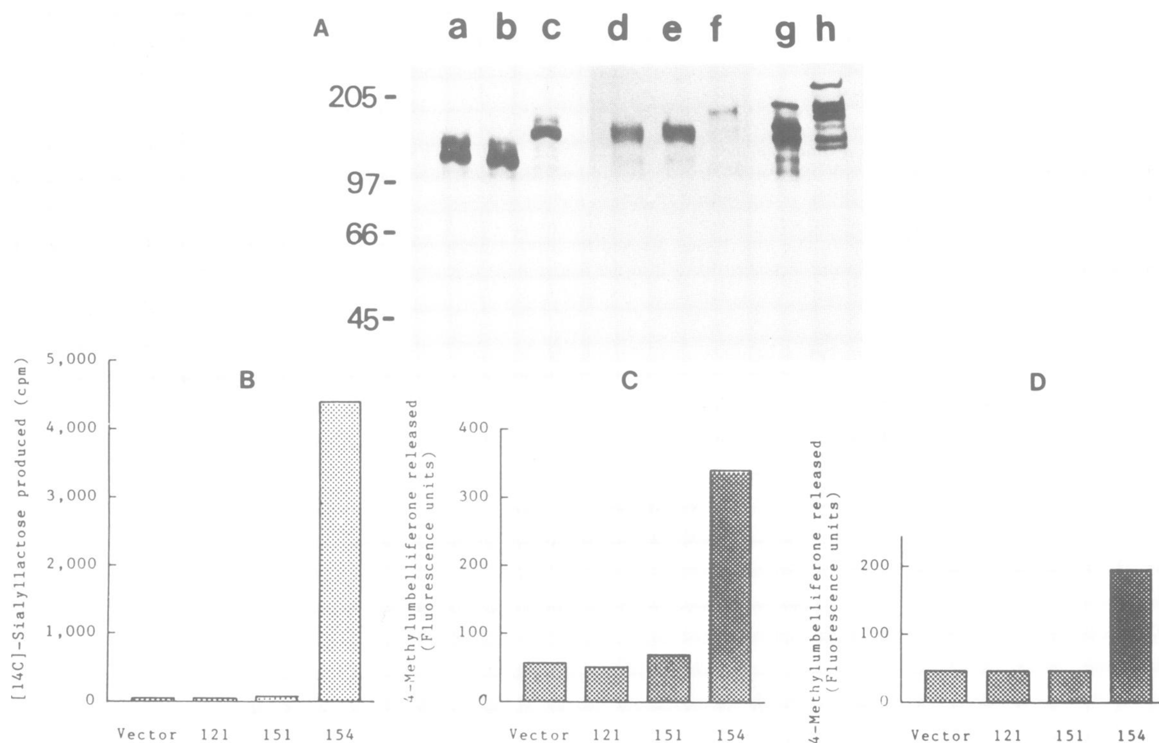

Fig. 5. Immunoblot and expression analysis of *trans*-sialidase gene products from COS cells. **A.** The three members of the *trans*-sialidase gene family were expressed in COS-1 cells using the vector pCDM-8. Three days after transfection, culture supernatants and cell extracts were analysed for the presence of *trans*-sialidase protein by fractionation on SDS−PAGE gels, transfer to nitrocellulose filters and probing with mAb 39. Lanes a, b and c, extracts of cells transfected with clones 121, 151 and 154; lanes d, e and f, culture supernatants from cells transfected with clones 121, 151 and 154; lane g, total trypomastigote extract; lane h, trypomastigote culture supernatant. **B.** *Trans*-sialidase activity of the transfectants was detected by incubating 10μl of a 100 μl total cell extract for 2 h in a *trans*-sialidase reaction. **C.** 10 μl of the same extracts were incubated for 5 h to detect neuraminidase activity. **D.** Neuraminidase in the culture supernatant from the transfected cells was detected by incubating 10 μl of the 20 ml culture fluid for 5 h in a neuraminidase reaction.

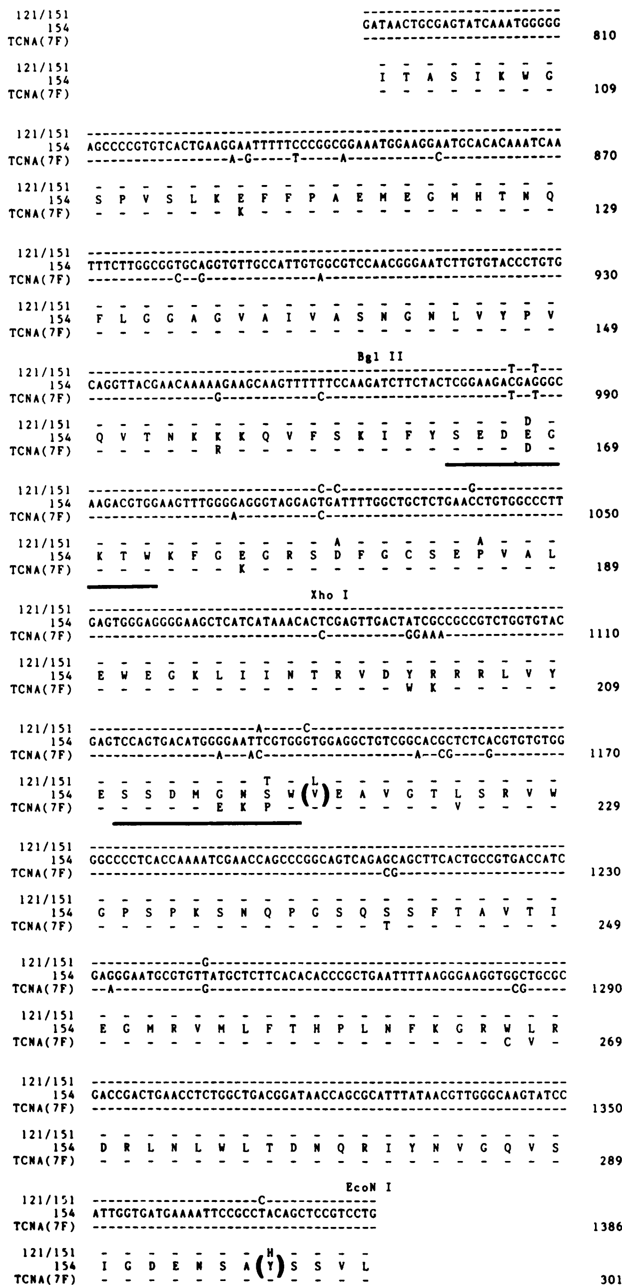

Fig. 6. Comparison of nucleotide and amino acid sequences of active and inactive *trans*-sialidase–neuraminidase genes. A region encompassing the *Bgl*II to *Eco*NI sites from clones 121, 151 and 154 was sequenced and aligned with the same region from the enzymatically active neuraminidase gene TCNA(7F). A dash indicates that the nucleotide or amino acid is the same as in clone 154 and substitutions are indicated. Nucleotide and amino acid numbers are according to the TCNA(7F) sequence (Pereira *et al.*, 1991). The recognition sequences for restriction enzymes *Bgl*II, *Xho*I and *Eco*NI are indicated above the nucleotide lines. The two consensus SXDXGXTW sequences in this region of the genes are underlined. The two amino acid positions, (in the *Xho*I to *Eco*NI region demonstrated to be required for activity), which distinguish the active from the inactive genes are shown in brackets.

mapping prior to transfection. Culture supernatants and extracts of washed cell pellets were both examined for the presence of protein and enzyme activities.

Figure 5A shows that the proteins made in COS-1 cells display the same relative size differences between 121/151 and 154 as the corresponding products from *E.coli*. The banding pattern, however, is now less complex, with one major product and several distinct minor products (lanes a, b and c). The products isolated from the culture medium of the transfected COS cells migrate with apparent higher molecular weight on SDS–PAGE than those isolated from cellular extracts (lanes d, e and f). Identical results were obtained when the 'shed', i.e. found in the culture medium, and cell-associated products of the parasite itself were examined, (lanes g and h).

Extracts of COS-1 cells transfected with clone 154 expressed *trans*-sialidase and neuraminidase activities, and culture supernatant from these cells also expressed neuraminidase activity, (*trans*-sialidase activity was not measured). The clone 121 and 151 transfectants expressed neither activity, (Figure 5B, C and D).

Sequence comparison between active and inactive trans-sialidase – neuraminidase genes

The restriction mapping, oligo hybridization and expression experiments in *E.coli* and COS-1 cells all indicated that multiple sequence differences lie between the *Bgl*II and *Eco*NI sites, some of which are responsible for the different enzyme activities in clones 121, 151 as compared with 154. Accordingly, the sequences of the *Bgl*II to *Eco*NI fragments from all three clones were determined. A comparison of the sequences of these fragments with neuraminidase is shown in Figure 6. The clones 121 and 151 are identical between the *Bgl*II and *Eco*NI sites and differ from 154 in nine nucleotide positions, six of which result in amino acid changes. Four of these distinguishing amino acids are found in the enzymatically active neuraminidase gene of Pereira *et al.* (1991).

Discussion

Trans-sialidase and neuraminidase activities are stage-specific and expressed on the surface of *T.cruzi* trypomastigotes. Both activities modulate parasite invasion of mammalian host cells (Cavallesco and Pereira, 1988; Prioli *et al.*, 1990; Schenkman *et al.*, 1991a,b). Here we demonstrate that these two sialic acid related enzyme reactions are catalysed by the product of a single gene and presumably a single protein, from *T.cruzi*. As proposed for other *trans*-glycosidases, the *trans*-sialidase–neuraminidase may bind to a sialic acid donor molecule and form a sialylated enzyme intermediate and then the bound sialic acid is transferred to acceptor saccharides or in their absence, to water molecules (Hassid and Neufeld, 1962).

Monoclonal antibodies against *trans*-sialidase and neuraminidase detect a group of molecules, migrating from 120–220 kDa on SDS–polyacrylamide gels (Schenkman *et al.*, 1992). The precise relationship between the multiple bands observed on these Western blots of parasite extracts and those from extracts of *E.coli* or COS cells expressing single genes is unclear. The results shown in Figure 1 indicate that one source of the size heterogeneity of proteins isolated from parasites is probably to be the expression of multiple members of the gene family that differ in the length of the C-terminal repeat-containing region. We identified at least three types of genes with respect to the length of this region of the gene. Clones 121, 151 and 210 contain 0.9 kb of repeat region, 154 contains 1.2 kb and 150 has >1.5 kb. The length polymorphism is also detected by Southern hybridization of Y strain (data not shown) and Silvio X-10/4

clone (Pereira *et al.*, 1991) genomic DNA. Similar long terminal repeat units are also found in the C-terminal region of shed acute phase antigen (SAPA) of *T.cruzi*, whose N-terminal amino acid sequence has been shown to be 80% identical to neuraminidase (Macina *et al.*, 1989; Pereira *et al.*, 1991; Parodi *et al.*, 1992). Loci containing SAPA genes, as defined by a probe encoding the C-terminal repeats, were shown to be distributed amongst several chromosomes and different genes contain variable numbers of repeat units (Macina *et al.*, 1989; Henriksson *et al.*, 1990). Furthermore, different size mRNAs, which reflect variable numbers of C-terminal repeat units, are detected in trypomastigotes (Macina *et al.*, 1989), suggesting that multiple family members are simultaneously transcribed.

These results, however, do not explain the multiple protein bands obtained when the individual genes are expressed in bacteria or mammalian cells. The extent of heterogeneity of the bacterial products suggests that proteolysis of the protein products is occurring. It can be assumed that carbohydrate addition is not contributing to product heterogeneity in this system (nor it follows, is carbohydrate addition necessary for enzymatic activity). The simpler banding pattern of the COS cell products suggests that most of these problems have been circumvented, but they also demonstrate that a single gene can yield multiple protein products. When clone 154 was expressed in bacteria, enzymatic activities were only detected when the largest band shown in Figure 2 (lane c) was also evident. The lack of activity of products from clones 121 and 151 are probably not due to sequence-specific higher levels of degradation since protein products of the hybrid constructs (both enzymaticaly active and inactive) did not show increased levels of degradation (data not shown).

There are also differences in the SDS−PAGE migration rates of products isolated from COS cell extracts and those found in the culture medium of these cells, (Figure 5A). A similar shift in apparent molecular weight is seen when comparing *trans*-sialidase found in extracts of parasites with *trans*-sialidase isolated from the parasite culture medium. *Trans*-sialidase−neuraminidase is anchored in the parasite membrane by a glycolipid tail, (Rosenberg *et al.*, 1991) and these size differences may reflect differences in gel migration of the membrane-bound (anchored) and the shed (cleaved) forms of the enzyme.

The genomic clones obtained here differed not only in the length of the C-terminal repeat region, but also in their enzyme activities when expressed in either bacteria or COS cells. The enzyme activity analysis of reconstructed plasmids demonstrated that coding sequences that lie between the *Xho*I and *Eco*NI sites are necessary for complete enzyme activities, since only those recombinants containing this fragment from clone 154 were active. This fragment encodes only one of the SXDXGXTW consensus boxes (amino acids 211−218, Figure 6) found to be conserved in various neuraminidases.

Clones 121/151 and 154 are predicted to differ from one another at three amino acid positions in the *Xho*I to *Eco*NI coding region and two of these three amino acids, valine and tyrosine, are conserved between clone 154 and the enzymatically active neuraminidase clone 7F of Pereira *et al.* (1991). Val219 in the active clones 154 and 7F is replaced by leucine in the inactive clones 121/151, and Tyr297 of the active clones is substituted with histidine in the inactive clones. The valine/leucine substitution may not be of relevance, since they are chemically similar amino acids, and the amino acid residue of *Clostridium perfringens* neuraminidase corresponding to position 219 is not conserved between the *C.perfringens* and *T.cruzi* enzymes. On the other hand, Tyr is found in the bacterial neuraminidase at the position corresponding to Tyr297 of *T.cruzi* neuraminidase (Pereira *et al.*, 1991) and the substitution of tyrosine for histidine at this position in clones 121/151 may eliminate enzyme activity through local charge or conformational alterations. Site-directed mutagenesis experiments to test the role of the amino acid differences between the active and inactive gene products are underway.

The apparent presence of genes in the *trans*-sialidase−neuraminidase gene family that encode products lacking enzymatic activity raises the question of the role of these proteins and establishes two classes of products from this gene family, those with and those without enzymatic activites, both of which bear highly antigenic C-terminal repeat units. The relatively small number of nucleotide changes (and even smaller number of amino acid changes) and retention of the complete ORF, argues for a specific role for these gene products. At this time, however, there is no good estimate of the level of expression (at either the transcriptional or protein levels) of the inactive genes, nor of any particular active gene, due to the fact that the genes were isolated from a genomic library and to the difficulty in designing probes which detect products of the two gene types.

As previously mentioned, another trypomastigote product, SAPA, also bears extensive homology to *trans*-sialidase−neuraminidase and as such represents a member of the *trans*-sialidase−neuraminidase gene family. Antisera that recognize SAPA have been used to immunoprecipitate both enzymatic activities and while the cloning of a gene encoding SAPA has been described (Parodi *et al.*, 1992), no enzymatic activities of its gene product have been directly demonstrated.

A distinct gene family encoding trypomastigote-specific 85 kDa surface antigens with significant sequence homology to the *trans*-silaidase/neuraminidase has been described in *T.cruzi* (Peterson *et al.*, 1989; Takle and Cross, 1989; Kahn *et al.*, 1990, 1991; Takle *et al.*, 1991). The 85 kDa antigens lack the C-terminal repeats but contain the consensus motif SXDXGXTW found in the *trans*-sialidase−neuraminidases and in SAPA (Pollevick *et al.*, 1991), which is also shared with bacterial neuraminidases. However, in spite of this homology, to date no enzymatic activity has been detected in the translation products of any the 85 kDa antigen genes. Until an enzymatic activity or other role of these gene products is demonstrated, the 85 kDa antigens would seem to represent a distinct class of *T.cruzi* antigens, with limited structural similarities restricted to the N-terminal half of the *trans*-sialidase−neuraminidase proteins.

Another feature of the *trans*-sialidase genes cloned here is the probable tandem arrangement of at least some of the genes in the parasite genome. The original lambda clones, numbers 127 and 130 (Figure 1), both contain what appears to be the 3′ end of one gene followed by the 5′ end of another. This arrangement of tandemly duplicated genes is not uncommon in kinetoplastids, as was shown for cruzipain genes in *T.cruzi* (Campetella *et al.*, 1992), tubulin genes in *T. brucei* (Thomashow *et al.*, 1983) and gp63 in *Leishmania major* (Medina-Acosta *et al.*, 1989). If the same type of arrangement is shown for the *trans*-sialidase−neuraminidase genes, it would be of interest to determine whether the

tandem groups of genes represent homogeneous arrays of enzymatically active or inactive genes or alternatively, each group contains a mixture of active and inactive genes.

Materials and methods

Parasites

The Y strain of *Trypanosoma cruzi* (Silva and Nussenzweig, 1953) was used in this study. Epimastigotes were grown in liver infusion medium containing 10% fetal bovine serum. Tissue culture trypomastigotes were obtained from infected LLC-MK 2 cells as described (Schenkman *et al.*, 1992). Parasites were harvested from culture supernatants and stored at −70°C after washing in phosphate buffered saline, pH 7.2, (PBS), containing 20 mM glucose. Supernatants collected from trypomastigote cultures were stored at −20°C.

DNA isolation from Trypanosoma cruzi

Epimastigote pellets were suspended in NET buffer (20 mM Tris pH 8.0, 50 mM EDTA and 100 mM NaCl) at a ratio of 1 ml buffer/0.1 g cell pellet and SDS was added to a final concentration of 1%. The suspension was treated with proteinase K (100 μg/ml) for 3 h at 50°C, followed by phenol and phenol−chloroform extractions. The DNA collected by ethanol precipitaion was dissolved in TE (10 mM Tris, 1 mM EDTA pH 8.0) and stored at 4°C. Restriction enzyme digestion and Southern hybridization analysis were performed using standard methods (Sambrook *et al.*, 1989).

Genomic DNA library construction

25 μg of total DNA was sheared by passing it 300 times through a 27 gauge needle, followed by treatment with 10 units of T4 DNA polymerase in the presence of four deoxynucleoside triphosphates at 50 μM each for 1 h at room temperature. After methylation of *Eco*RI sites, DNA was fractionated on a 10−40% sucrose gradient by centrifugation. *Eco*RI linker was added to the 5−9 kb DNA fragments and excess linker was removed by centrifugation after *Eco*RI treatment. 0.5 μg DNA was ligated with 1.0 μg of *Eco*RI, phosphatase treated lambda ZAPII vector (Stratagene, La Jolla, CA) and packaged to obtain a 5.5×10^5 p.f.u. stock *T.cruzi* genomic library.

Genomic library screening

To obtain *trans*-sialidase DNA clones the unamplified genomic library was screened with ^{32}P radiolabelled oligonucleotides representing C- and N-terminal encoding sequences of the *T.cruzi* neuraminidase gene (Pereira *et al.*, 1991). Oligo NA-C1 (33mer, 5′-GACACCAGTGCCCACAGTACG-CCCTCGACTCCC-3′) contains the codons for the conserved 11 amino acids in the 12 amino acid C-terminal repeat unit. NA-N2 (24mer, 5′-TCGG-AAGATGATGGCAAGACGTGG-3′) represents one of the consensus sequences (S-X-D-X-G-X-T-W) that are conserved in bacterial and viral neuraminidases and are also found in *T.cruzi* neuraminidase (Pereira *et al.*, 1991). The phage hybridizing to either oligonucleotide were spotted onto a lawn of *E.coli* XL-1 Blue and induced for protein synthesis for 4 h by overlaying a nitrocellulose filter that had been soaked in 10 mM IPTG. The filter was then incubated with anti-*trans*-sialidase mAb 39 (Schenkman *et al.*, 1991b), followed by rabbit anti-mouse alkaline phosphatase and substrate. Nine strong antibody-positive clones were identified, all of which also bound the NA-C1 oligo.

Expression of trans-sialidase genes in E.coli

The nine antibody-positive lambda clones were converted into pBluescript SK(−) plasmid form using R408 helper phage according to instructions supplied by Stratagene. Overnight cultures of *E.coli* XL-1 Blue containing the plasmids were diluted 1:50 with fresh Luria broth supplemented with 12.5 μg/ml tetracycline, 100 μg/ml ampicillin and 0.02% glucose and grown for 4 h at 37°C. Protein synthesis was induced by adding the same volume of Luria broth containing 2 mM IPTG and incubating for another 2 h. The bacterial pellets were collected by centrifugation at 3000 *g* for 15 min, washed with PBS and resuspended in 1:100 of the original culture volume using 50 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP-40 and proteinase inhibitors (1 mM PMSF, 5 μg/ml pepstatin, antipain and leupeptin and 4 mM iodoacetamide). The cells were treated with lysozyme (1 mg/ml) for 10 min at 0°C and then with DNase I (10 μg/ml) for 10 min at 37°C. Cell debris was removed by centrifugation in a microcentrifuge for 10 min after adding EDTA to 10 mM and supernatants were stored at −20°C. Plasmids from clones designated 121, 151 and 154 were modified for expression by removing a large part of the 5′ untranslated region by digestion of the plasmids with *Sac*II and religation.

Expression of trans-sialidase genes in COS cells

The genes from the modified versions of clones 121, 151 and 154 were prepared for insertion into pCDM-8 (Aruffo and Seed, 1987) by digestion of pBS−*trans*-sialidase constructs with *Sac*II, elimination of the overhang with T4 DNA polymerase and digestion with *Sal*I, which cuts 3′ of the gene in the pBS polylinker. The modified and released insert fragment was inserted into pCDM-8, which had been digested with *Hin*dIII and filled in with DNA polymerase I and subsequently digested with *Xho*I. The resulting expression plasmids were transfected into COS-1 cells using Lipofectin (BRL/Life Technologies, Inc., Gaithersburg, MD) as follows: 10 ml of serum-free DMEM medium containing 10 μg of CsCl-purified plasmid DNA and 30 μg Lipofectin were added per 100 mm^2 semi-confluent plate of COS-1 cells. Cells were incubated for 10 h at 37°C and 10 ml of new medium containing 20% fetal calf serum was added. After another 60 h incubation, transfected cells and culture supernatants were analysed for expression by western blotting and *trans*-sialidase enzyme assay.

Enzyme assays

Trans-sialidase activity was detected by incubating enzyme-containing solutions in 20 mM HEPES pH 7.2, containing 1 mM sialyllactose (50 nmol) and 25 000−40 000 c.p.m. of [D-glucose-l-^{14}C]lactose (0.4 nmol, Amersham) as previously described by Schenkman *et al.* (1991a). Neuraminidase activity was analysed by hydrolysis of the fluorogenic substrate 2′-(4-methylumbelliferyl)α-D-*N*-acetylneuraminic acid (4MU-NANA, Sigma) as described by Schenkman *et al.* (1992). The reaction was performed in a total volume of 20 μl containing 0.1 M sodium phosphate buffer pH 6.5 and 0.2 mM 4MU-NANA and stopped by adding 200 μl of 0.2 M Tris pH 9.5.

DNA sequencing

Plasmid DNA was prepared by alkaline lysis and nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using Sequenase (United States Biochemicals, Cleveland, OH).

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