Tumorigenic Poxviruses: Characterization of the Expression of an Epidermal Growth Factor Related Gene in Shope Fibroma Virus

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Received May 17, 1990; accepted August 1, 1990

The transcription and translation of an epidermal growth factor (EGF) related gene in the Leporipoxvirus Shope fibroma virus (SFV), termed the Shope fibroma growth factor (SFGF), have been characterized. Three early RNA transcripts complimentary to an anti-SFGF oligonucleotide were detected by Northern blot analysis, while no late transcripts were expressed. The activity of the SFGF early promoter was measured using a transient gene expression assay in SFV-infected cells using the bacterial chloramphenicol acetyltransferase as a reporter gene. Deletion analysis showed that the functional SFGF promoter domain is an AT-rich sequence contained within 30 bp of the major transcriptional initiation site as is typical of early poxvirus promoters. An intracellular form of the SFGF gene product was immunoprecipitated from infected lysates using rabbit antiserum raised against a synthetic SFGF (amino acids 26-80). A 16-kDa product was detected, while in cells infected in the presence of tunicamycin, the immunoprecipitated product had a mobility on SDS-polyacrylamide gels of approximately 6 kDa, indicating that the SFGF gene product is extensively post-transcriptionally modified. The intracellular 16-kDa form can be pulse-chased to a 14-kDa form but the secreted form of SFGF could not be detected in the medium using this anti-peptide antiserum.

Although the mechanism of tumor formation in SFV-infected animals is not yet clarified, Kato et al. (3) proposed that a growth promoting factor of either cellular or viral origin is released from the SFV-infected cells and induces the neighboring uninfected fibroblasts to proliferate. This proposal was further strengthened when it was found that both the Orthopoxvirus vaccinia and the Leporipoxvirus SFV contain genes which encode an epidermal growth factor (EGF) related protein (4-9). The vaccinia growth factor gene (VGF) is transcribed at early times (10), and the gene product has been purified from the medium of infected cells. Amino acid sequence analysis of the secreted VGF and immunoprecipitation using anti-VGF peptide-specific rabbit antiserum has demonstrated that VGF is initially synthesized as an N-linked glycosylated membrane-associated precursor which is cleaved and finally released as a secreted molecule. Although vaccinia virus does not induce tumors, Buller et al. (12, 13) found that when the VGF gene was inactivated by targeted inser- tional mutagenesis, the VGF- virus was less patho- genic as measured by intracranial injections in mice. Furthermore, when inoculated onto the chorioallantoic membrane of fertilized chicken eggs, the deletion variant induced less proliferation of the surrounding uninfected ectodermal and endodermal tissues than did the wild-type virus (12, 13).

As a first step in investigating the function of the Shope fibroma virus growth factor (SFGF) in a hinogy of a prototype tumorigenic poxvirus, we have examined the expression of this gene at the transcriptional and translational levels.

Northern blots of total cellular RNA from SFV-infected RK-13 cells were hybridized with a 32P-end-labeled oligonucleotide probe complimentary to the
SHORT COMMUNICATIONS

FIG. 1. Northern blot analysis of SFGF transcripts from SFV-infected RK-13 cells. RK-13 cells were infected with SFV and total cellular RNA was extracted at various times postinfection (p.i.) as indicated. Total RNA (20 μg) was electrophoresed on a 1% formaldehyde agarose gel, transferred to a nylon membrane (N-Hybond), and probed with an antisense SFGF 32P-end-labeled oligonucleotide (5'-TTGTAACGAGAAGAGTTATTATGCAGATATACGTATTATCAATGGCATGGTTATGCACTTAATA-3'). The sizes of the RNA markers are indicated at the left of the figure.

SFGF gene, and three major RNA species ranging in size from 2.4 to 3.2 kbp were detected, with the smallest species being the most abundant (Fig. 1). All three species were detected at 2, 4, and 6 hr, but not at 24 hr postinfection, consistent with these being early mRNA transcripts. Although the function of early promoter sequence has been conserved between the lepori- and the ortho-poxviruses (14), the timing of the switch between early and late gene expression has not. Early transcription occurs prior to DNA synthesis, which in vaccinia is at 2–3 hr postinfection (15), whereas in SFV it is at 8–9 hr postinfection (16). Thus, at 6 hr postinfection early transcripts are abundant in SFV-infected cells and absent in vaccinia-infected cells (14). It is possible that either all three species are 5'-coterminal SFGF mRNA, with the larger two species occurring as a result of read-through of early transcriptional termination signals as is common among early poxvirus genes, or they may represent RNAs initiating from the promoter of the upstream and overlapping ORF T1-L. In either case, it is clear that all the species are expressed as early RNAs only.

A second antisense SFGF oligonucleotide was hybridized to total early RNA from SFV-infected cells, and the hybrids were extended with reverse transcriptase, treated with RNase A, and then electrophoresed on an 8% sequencing gel (Fig. 2). Of the two extended products observed, the major product occurs at a thymine in the sense sequence 75 nucleotides upstream of the initiating AUG codon for the ORF and a second minor extended product corresponds to a C residue three nucleotides upstream of the major start site. Relatively few early poxvirus transcripts have 5' ends which map to pyrimidines. Given the fact that Boone and Moss (17) found that the initiating position of bulk early RNA consisted of 55% G and 45% A residues, while Davisson and Moss (19) have shown that substituting a pyrimidine for the initiating purine residue results in a change of initiation sites to the next purine, it is possible that these designated pyrimidine residues are not the true sites of initiation of transcription for these RNA species. For example, the structure of the cap of the RNA itself could affect the precise mapping of the 5' end of the transcript. If the reverse transcriptase did not fully extend the primers and failed to copy the last nucleotide then the initiating nucleotide for both transcripts would be a purine (A).

In the S1 analysis of Macaulay et al. (18), this SFGF transcript was detected only at late times. Further work has shown that the SFV infections used in that work were not of sufficiently high m.o.i. to ensure that a second round of infections could not occur at late times of infection. For example, S1 analysis of the T1-ORF in SFV originally indicated that the identical start site was...

FIG. 2. Determination of the 5' transcriptional start of the SFGF gene by primer extension analysis. A 32P-end-labeled oligonucleotide (5'-TAGAGGCACACTAGGT-3') was hybridized to total RNA (10 μg), from mock- or SFV-infected RK-13 cells (4 hr postinfection). The products of the reverse transcriptase reaction were analyzed on an 8% urea-acrylamide gel beside a dideoxy sequencing reaction using the same oligonucleotide primer. The sense sequence on the right illustrates the position to which the major (T) and minor (C) extended products (arrows) correspond.
used at both early and late times. However, we later showed that at higher multiplicities the T1 promoter is clearly shut off at late times (14). In the work presented here the m.o.i. was 10 and no late transcripts were detected by Northern blot analysis at late times (Fig. 1) or by primer extension analysis (not shown), thus supporting the contention that SFGF is a bona fide early gene.

To characterize the 5' sequence boundaries of the SFGF early promoter the minimal upstream sequences which allow the promoter to function in a transient CAT gene expression assay were determined (20). The putative SFGF early promoter was first isolated on a 372-bp BglI–HincII DNA fragment spanning the region −336 to +36 relative to the transcriptional initiation site and cloned into a CAT expression vector to create pSFV-CAT. This clone was then used to generate a series of 5' deletions that span the putative promoter sequence and several of these were then used as substrates for oligonucleotide-directed mutagenesis. These various constructs and their activities in the transient CAT expression assays are shown in Fig. 3.

Deletion of the sequences between −336 and −43 did not significantly alter the amount of CAT activity (Fig. 3, upper panel), and further deletion from −43 to −25 reduced CAT activity to 77% that of the full-length clone (Fig. 3, Del. −25). However, deletion of the AT-rich region between −25 and −15 completely eliminated CAT activity (Fig. 3, Del. −15). When an 18-bp oligonucleotide containing the sequences between −43 and −25 was inserted into the Del-15 construct to give Mut-1, only 12% of the CAT activity was regenerated, showing that sequences between −25 and −15 define the 5' boundary of the SFGF promoter. The importance of this region is demonstrated by the Mut-2 construct in which the sequence between −25 and −15 is inserted adjacent to the 5' initiation site. This clone is able to restore 65% of the wild-type activity indicating that the sequences deleted by Del-15 are sufficient to provide substantial promoter function. The sequences between −14 and +1 were able to augment activity only by a further 13% to 77% (Fig. 4, Del. 25). Within the SFGF promoter is an A-rich octanucleotide that is present as two partially overlapping direct repeats between −23 and −10 (underlined in Fig. 3B). The importance of these A residues is demonstrated by the Mut-3 construct, where two A residues are inserted into Del-15 to reconstruct the octanucleotide, in which CAT expression is increased from 2 to 72% of wild-type levels. These results are consistent with previous findings that A-rich regions are important early promoter elements (14, 19, 21).

To analyze the SFGF gene product we utilized a rabbit antiserum raised against a synthetic peptide SFGF26-80 that contains the primary translated SFGF amino acid sequences from isoleucine (26) to tyrosine (80) (22). When this peptide is correctly folded it is biologically active in growth factor assays and it competes with EGF for binding to the EGF receptor (22, 23).

Western blot analysis was used to successfully demonstrate the reactivity of the rabbit antiserum toward both the synthetic peptide and a bacterial fusion protein consisting of the 37-kDa N-terminal portion of the Escherichia coli trp E protein fused to the entire 80-aa coding sequence of SFGF in a pATH expression vector. However, all attempts using Western blot analysis to detect SFGF in lysates or in the conditioned medium of SFV-infected cells with this anti-peptide antibody have been negative (not shown).

To increase the sensitivity of detection we attempted
were pelleted in a microfuge. The beads were washed with RIPA then diluted with RIPA (minus SDS) to give a final SDS concentration.

Tunicamycin (10 μg/ml) was added and incubated on ice for 30 min, and the Sepharose beads pulsed with [%35S]cysteine for 15 min, and chased with normal medium for the various times indicated. Tunicamycin (10 μg/ml) was used to block N-terminal glycosylation in lanes 1 and 2. Antiserum preadsorbed with the SFGF26-80 peptide was used in the even-numbered lanes, while untreated antiserum was used to immunoprecipitate SFGF. Molecular weight markers are indicated to the left of the gel. The infected cells were homogenized in RIPA buffer supplemented with sodium dodecyl sulfate (SDS) to 5% (150 mM NaCl, 10 mM Tris, pH 7.5, 1% Triton X-100, 1% Na-deoxycholate, and 5% SDS). This lysate was boiled for 5 min to denature the protein and then diluted with RIPA (minus SDS) to give a final SDS concentration of 1%. Anti-SFGF26-80 antiserum was added to a final dilution of 1:100 and incubated at 4°C for 1 hr, protein A-Sepharose was added and incubated on ice for 30 min, and the Sepharose beads were pelleted in a microfuge. The beads were washed with RIPA minus SDS and then dissolved in Laemmli gel loading buffer (27).

Fig. 4. Pulse-chase analysis of SFGF. Total cell lysates were prepared from RK-13 cells that were infected with SFV (m.o.i. 20), pulsed with [35S]cysteine for 15 min, and chased with normal medium for the various times indicated. Tunicamycin (10 μg/ml) was used to block N-terminal glycosylation in lanes 1 and 2. Antiserum preadsorbed with the SFGF26-80 peptide was used in the even-numbered lanes, while untreated antiserum was used to immunoprecipitate SFGF. Molecular weight markers are indicated to the left of the gel. The infected cells were homogenized in RIPA buffer supplemented with sodium dodecyl sulfate (SDS) to 5% (150 mM NaCl, 10 mM Tris, pH 7.5, 1% Triton X-100, 1% Na-deoxycholate, and 5% SDS). This lysate was boiled for 5 min to denature the protein and then diluted with RIPA (minus SDS) to give a final SDS concentration of 1%. Anti-SFGF26-80 antiserum was added to a final dilution of 1:100 and incubated at 4°C for 1 hr, protein A-Sepharose was added and incubated on ice for 30 min, and the Sepharose beads were pelleted in a microfuge. The beads were washed with RIPA minus SDS and then dissolved in Laemmli gel loading buffer (27).

No polypeptide was specifically immunoprecipitated by the antiserum using standard conditions (not shown). In view of the fact that the antiserum recognizes both the denatured fusion protein and the synthetic SFGF26-80 by Western blot analysis, we modified the immunoprecipitation procedure by first boiling the cell lysate in 5% SDS and then adding the antiserum to the presence of 1% SDS to see if the denatured form of SFGF would be recognized. A 16-kDa polypeptide could now be specifically immunoprecipitated from SFV-infected cells (Fig. 4, lane 3), and this immunoprecipitation was blocked by preincubating the antiserum with the synthetic SFGF26-80 (Fig. 4, lane 4).

The calculated molecular weight of the full-length SFGF is 10,974 Da and, with the removal of a putative 19-amino-acid hydrophobic signal sequence, would be reduced to 8575 Da. Given that there is a difference between this calculated molecular weight and the observed mobility of immunoprecipitated SFGF, we investigated whether SFGF might be glycosylated similarly to VGF (8, 11), by preincubating the cells in tunicamycin, which inhibits N-linked glycosylation (Fig. 4, lanes 1 and 3). A broad 6-kDa band is specifically immunoprecipitated in the presence of the inhibitor, which is much closer to the calculated molecular weight of the SFGF reading frame.

Pulse-chase analysis (Fig. 4, lanes 3–14) shows that a 16- and an 18-kDa band are recognized by the SFGF26-80 antibody. Although the 16-kDa band is chased to a 14-kDa form we have been unable to detect secreted SFGF by immunoprecipitation with this synthetic peptide antibody. We interpret these results to indicate that, like VGF, SFGF is an early viral gene product that is post-translationally modified by glycosylation with the possible removal of a putative hydrophobic N-terminal signal sequence to give a final 16-kDa form as the most abundant form within the infected cell. This 16-kDa form is further processed to give a 14-kDa intracellular form; however, which of these forms is actually secreted will require analysis by antibody reagents which recognize the native conformation of the mature growth factor (in progress).

Inspection of the SFGF sequence (11) shows that, unlike other members of the EGF family, including VGF (8), EGF (24), and TGF-α (25, 26) SFGF is not first made in a pre–pro-form, and there is no obvious C-terminal putative membrane spanning hydrophobic domain. Therefore, our inability to detect a secreted form of SFGF with the synthetic peptide antibody may be due to the fact that it is relatively cell associated or, alternatively, that this antiserum does not recognize the final peptide product because post-translational processing masks the major antigenic site. An activity which competes with EGF for binding to its receptor has been detected in both SFV-infected cell lysates and conditioned medium (W. Chang, unpublished results), but because the levels of production of SFGF protein are so low it has not been practical to purify and sequence this activity directly as was done with VGF (8).

One way to address the question of the role of SFGF in infected host tissues would be to inactivate the SFGF gene by the targeted insertion of a marker gene. Preliminary studies on the function of such deletion mutations of EGF-related genes in several Leproripoxviruses indicates that this gene family is not solely responsible for the proliferative response at the site of infection but their deletion profoundly affects the virulence of the viruses in the infected animal (Opgenorth et al., in preparation). The analysis of the role of these growth factors in the pathogenesis of the tumorigenic poxviruses should shed light on the biological functions of other members of the EGF receptor ligand family as well.

ACKNOWLEDGMENTS

We thank Scott Austin for preparing oligonucleotides, Paula Traktman for the pATH expression vector, and S. Kasinec for help with the...
manuscript preparation. G.M. and C.M. are supported by the Alberta Heritage Foundation for Medical Research and the NCI of Canada.

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