Isolation of a rearranged human transforming gene following transfection of Kaposi sarcoma DNA

(NIH 3T3 transfection/molecular cloning/c-fms protooncogene)

PASQUALE DELLI BODI* AND CLAUDIO BASILICO

Department of Pathology, New York University School of Medicine, 550 First Avenue, New York, NY 10016

Communicated by Hidesaburo Hanafusa, May 4, 1987 (received for review March 3, 1987)

ABSTRACT By transfecting high molecular weight DNA from a Kaposi sarcoma lesion into murine NIH 3T3 cells, we have identified and molecularly cloned a set of human DNA sequences capable of inducing focus formation, growth in agar, and tumorigenicity in these cells. The human DNA sequences present in primary, secondary, and tertiary NIH 3T3 transformants encompass about 32 kilobases (kb) and contain four rearrangements with respect to normal human DNA and a portion of the c-fms protooncogene (FMS in human gene nomenclature). However, the minimal transforming region (6.6 kb) identified in our cloned DNA borders on the c-fms DNA region but does not contain c-fms coding sequences. The c-fms sequences are also not represented in the two transcripts (≈1.2 and 3.5 kb) detected in NIH 3T3 transformants; however, they might provide elements regulating expression. Hybridization to several known oncogene probes and preliminary sequencing data indicate that we have identified a previously unrecognized “activated” oncogene. Since the rearrangements present in our cloned DNA sequences are not detectable in the original Kaposi tumor DNA used for transfection, it is possible that this oncogene was generated during gene transfer.

Kaposi sarcoma (KS) is a multifocal neoplastic disorder that appears with an unusually high frequency in acquired immunodeficiency syndrome (AIDS) patients, particularly the homosexual group, and in general in immunosuppressed individuals (1–4). Although KS is generally considered to be an angiosarcoma of endothelial/mesenchymal origin, its widely varied histopathology and generally slow clinical course, and the likelihood of polyclonal tumors has generated much controversy concerning its histogenesis and etiology (5).

Attempts to determine the presence of specific viral DNA sequences in the DNA of KS lesions has indicated that the AIDS retrovirus, cytomegalovirus (CMV), or hepatitis B virus are not responsible for the altered growth properties of KS cells (6). We have tried to determine whether a dominant transforming gene is present in KS DNA by transfecting DNA extracted from KS skin lesions of AIDS patients into NIH 3T3 cells and determining its ability to produce transformed foci. In one case we were able to obtain a focus of transformed cells, and the DNA from such a focus could transmit the transformed phenotype through successive transfection cycles. Molecular cloning of the human DNA sequences present in one of the secondary NIH 3T3 transformants revealed the transfer of ≈32 kilobases (kb) of human DNA including a transforming region of about 11 kb that contains at least two rearrangements with respect to normal human DNA and a portion of the c-fms protooncogene (FMS in human gene nomenclature).

MATERIALS AND METHODS

Cells and Transfection. Mouse NIH 3T3 and rat F2408 cell lines were cultured in Dulbecco’s modified Eagle’s medium containing 10% calf serum. DNA transfection of NIH 3T3 cells was performed by the calcium phosphate precipitation technique (8, 9); 24 hr after transfection, the cells were split 1:5 in medium containing 5% calf serum, and transformed foci were scored starting 2 weeks after transfection. For transfection with high molecular weight DNA, 50 μg of DNA per plate was used. When selection for resistance to G418 was applied, 2 μg of the pIW3-Neo plasmid (10) were cotransfected with high molecular weight DNA, and cells were incubated in medium containing 250 μg of G418 per ml (Geneticin, Gibco) starting 36 hr after transfection.

Nucleic Acid Purification and Blot Hybridization. High molecular weight DNA was prepared from KS necropsies or cultured cells as described (6, 11), digested with the appropriate restriction enzymes, size-fractionated on a 1% agarose gel, and transferred to nitrocellulose filters (12). Total RNA was extracted and purified by the guanidium isothiocyanate/cesium chloride method as described (13). Poly(A)+ RNA was selected by using the Amersham Hybond-mAP paper. RNAs were fractionated in the presence of formaldehyde by agarose gel electrophoresis and transferred to nitrocellulose filters as described by Maniatis et al. (14). Nucleic acid hybridization, washing, and autoradiography were performed as described (13).

Molecular Cloning. DNA was partially digested with Mbo I restriction enzyme as described by Maniatis et al. (14) and size-fractionated on an agarose gel. DNA of 16–22 kb was electroeluted and ligated to double-digested BamHI/EcoRI EMBL3 λ phage cloning vector (15). The ligated DNA was packaged in vitro by using Gigapack packaging (Stratagene Cloning Systems, San Diego, CA). Library screening was performed as described by Benton and Davis (16) with a probe for the highly human repetitive Alu I DNA sequences—the 0.3-kb BamHI fragment from the BLUR8 plasmid (17).

Recombinant Plasmids. The plasmids pCD(WII-1)1OA and pCD(WII-1)1OB were generated by ligating the 12-kb Sal I fragment from the recombinant phage WII-1 to an Xho I vector originating from the pCD Okayama and Berg (18) simian virus 40 (SV40) expression vector in both orientations with respect to the SV40 promoter. The plasmids pCD(WII-2)6.6A and pCD(WII-2)6.6B were generated by ligating the 6.6-kb Sal I fragment from the phage WII-2 to the same vector indicated above. The pGEM(WII-1)10 and pGEM(WII-2)6.6 plasmids were generated by inserting the 12-kb or 6.6-kb Sal

Abbreviations: KS, Kaposi sarcoma; AIDS, acquired immunodeficiency syndrome; Neo®, resistance to neomycin; SV40, simian virus 40; CMV, cytomegalovirus.

*On leave of absence from the Dipartimento di Genetica, Biologia Generale e Molecolare Facolta di Scienze, University of Naples, Italy.
I fragment from phages WII-1 or WII-2, respectively, in the Sal I site of the pGEM-3 plasmids vector (Promega Biotech, Madison, WI).

The following plasmids were generously provided by the indicated scientists: pBR322 from H. Hanafusa (Rockefeller University, New York); pSal1 (human NRAS cDNA), pBS-9 (subgenomic fragment of the Harvey murine sarcoma virus oncogene v-Ha-ras), pHiHi-3 (subgenomic fragment of the Kirsten murine sarcoma virus oncogene v-Ki-ras), pKW101 (subgenomic fragment of v-rel), pV-rf (subgenomic fragment of v-raf), pSM7C, and pSM3 (subgenomic fragments of feline sarcoma virus oncogene v-fms) from A. Pellicer (NYU Medical Center); pSMFeSV (v-fms) from C. J. Sherr (St. Jude Children’s Hospital); Hpc-fms-102 (human FMS cDNA) from I. Verma (Salk Institute); pV-Fos (v-fos) from E. Ziff (NYU); pMC413RC (c-myb), pSM-1 (human SIS cDNA), and pMYb (human MYC cDNA) from R. Dalla-Favera (NYU); pI76 (murine p53 cDNA) from R. Carroll (NYU); pHM2A (c-mos) and pHVP1, pHVP2, pHVP3, pHVP4, pHVP5, pHVP6, pHVP16, and pHVP18 (human papilloma viruses) from D. Morgan (NYU); pRL103 (human CMV-transforming region) from G. Hayward (Johns Hopkins University); and pARV-2 (AIDS retrovirus) from D. Dina (Chiron Corp).

RESULTS

High molecular weight DNAs extracted from several KS skin biopsies and necropsies were transfected into NIH 3T3 cells to detect whether they caused the appearance of foci of morphologically transformed cells. Whereas most of the DNAs tested gave negative results, one of them produced a distinct focus of highly refractile cells over the background of NIH 3T3 cells. After recloning in agar suspension medium, Southern blot hybridization (12) with the BLUR8 plasmid (17), which contains DNA sequences representative of the Alu I family of human repetitive DNA, revealed that all cells capable of growth in agar had acquired human DNA sequences (data not shown).

Cells from one agar colony were injected into nude mice (106 cells per mouse), and two of three mice developed tumors. DNA from one of the tumors (A15-I) appeared to have undergone amplification of human DNA sequences and was used to transfect NIH 3T3 cells together with a selectable marker, plasmid pI76 (10), which contains sequences conferring resistance to neomycin (Neo6) or G418 (19, 20). Selection for cells resistant to G418 revealed the presence of two colonies with a transformed morphology; selection for foci also resulted in two morphologically transformed foci.

DNAs extracted from the cells derived from two Neo6 transformed colonies and from foci were examined by blot hybridization with an Alu I DNA probe (17). The pattern of Alu I bands was much simplified with respect to the parental tumor, and showed common specific bands (data not shown).

DNA from one of the Neo6 colonies was used for a third cycle of transfection and again produced a small but significant number of Alu I-positive transformed foci. Thus, it appeared that we had obtained human DNA sequences capable of transforming NIH 3T3 cells, since the transformed phenotype correlated with the presence of human Alu I repetitive DNA. Therefore, we proceeded to precisely identify these sequences by molecular cloning.

Molecular Cloning. A genomic library of DNA extracted from one of the Neo6 secondary transformants (Neo-2) was constructed after Mbo I partial digestion and cloning into the EMBL3 λ phage vector (15). The library was screened for the presence of recombinant phages containing human Alu I DNA as described, and one recombinant phage (KS-2) was isolated. Hybridization with the Alu I probe and total mouse DNA revealed that it contained one Alu I sequence and two stretches of repetitive mouse DNA sequences and, thus, represented one of the junctions between mouse and human DNA in our Neo-2 transformant. We utilized several restriction enzyme fragments indicated in Fig. 1 to perform two rounds of chromosome walking" and isolated from the same library several recombinant phages that appeared to span the entire insertion of human DNA in the Neo-2 transformant. The restriction map of the human genomic DNA sequences present in these cells, as reconstructed from four overlapping recombinant phages among those isolated, is shown in Fig. 1. They encompass ≈32 kb of human DNA and contain three Alu I sequences. We used several DNA fragments derived from these sequences (Fig. 1) to determine their presence and arrangement in primary and secondary transformants as well as in normal human DNA. All of the sequences studied were present in the secondary and tertiary transformants, in the two primary tumors, and also in the DNA from the primary focus (Fig. 2).

Restriction enzyme analysis of the cloned human sequences revealed that they contained several rearrangements with respect to normal human DNA. Some of the evidence is shown in Fig. 2. DNAs from NIH 3T3, two secondary transformants, primary tumors, and normal human DNA were digested with BamHI, Xba I, and Sac I and blunt-hybridized to different probes derived from our recombinant phages. Probe B (Fig. 1) identifies two bands in the BamHI digests of both human and transformant DNA, but while the 6-kb band is the same in both transformant and human DNA, the 3-kb band present in the transformants is absent in normal human DNA, where a 4-kb band is detected. Therefore, a rearrangement must have occurred between the BamHI sites spanning this fragment. Probe C (Fig. 1) recognized the expected 6-kb band in Xba I-digested transformant DNA but recognized a band of higher molecular weight in human DNA. Probe G recognized two bands both in the transformant and in human DNA digested with Sac I, but only one of the bands was identical in the transformant and human DNA. Concordant results were obtained when the same probes were used with DNAs digested by different restriction enzymes, indicating that the results obtained are not due to restriction fragment length polymorphism. Similar evidence helped us to identify other DNA rearrangements at positions indicated on the map in Fig. 1.

Transcription of the Human DNA Sequences. We have used several of the DNA fragments indicated in Fig. 1 (A through I) as probes in RNA blot hybridization to determine whether the human sequences present in our transformants were transcribed into specific mRNAs. Probes A, B, C, D, and E did not identify any distinct mRNA species among poly(A)" RNAs extracted from primary or secondary transformants. However, probes F and G hybridized with two novel mRNA species of about 1.2 and 3.5 kb in primary and secondary transformants and also with some larger RNA species of variable length in some of the cell lines tested (Fig. 3). These probes did not detect any distinct RNA species in NIH 3T3 cells and only a faint band ≈4 kb long in the RNA extracted from human endothelial cells. Interestingly, probe H recognized only the longest mRNA but not the 1.2-kb species.

Since it is unlikely that the large probes A, B, and C consist entirely of intronic sequences, it appears that the transcribed regions in our human DNA sequences are restricted to about 12 kb of DNA. Since the 3.5-kb RNA species contains sequences not present in the 1.2-kb message, it is possible that the two mRNAs are produced by differential splicing or polyadenylation of a common precursor. The precise relationship between the two transcripts is being investigated through cDNA cloning. Preliminary results indicate that the polarity of transcription is for both mRNAs from left to right on the map of Fig. 1.

Presence of c-fms Sequences. We prepared radioactive probes corresponding to a number of viral and cellular
oncogenes and hybridized these probes to our phage DNA inserts to detect whether they contained regions of homology to these oncogenes. We detected no DNA sequence homology to the AIDS proviral DNA, CMV DNA, or several strains of human papillomavirus DNA. There was no hybridization with probes for the three ras oncogenes, myc, sis, erbB, rel, raf, myb, p53, mos, and fos, but a probe corresponding to viral v-fms c-DNA identified a region of strong homology included between a Sal I and the Sac I site at 33 kb on the map of Fig. 1. The use of subcloned fragments of the v-fms c-DNA (7, 21) as probes and hybridization of the phage DNA insert to the v-fms plasmids (7, 21) revealed that the

![Diagram of DNA sequences](image)

**Fig. 1.** Organization of the human DNA sequences inserted into the mouse genome in the Neo-2 secondary transformant, derived from the overlapping recombinant phages indicated in the figure. Segments A, B, C, D, E, F, G, H, and I represent DNA fragments derived from the phages shown and used in the characterization of these sequences by DNA and RNA blot-hybridization analyses as well as for mapping the DNA rearrangements. The probes C, D, E, and F contain moderately repetitive human DNA sequences. The interrupted lines indicate mouse DNA. The continuous dark lines indicate human DNA; the “V” in the straight line indicates the regions of joining between mouse and human DNA. Open boxes indicate regions containing mouse repetitive DNA. The hatched boxes indicate the regions containing the human Alu I repetitive DNA sequences. Squiggles indicate the approximate sites of DNA rearrangements. Restriction sites are indicated as follows: E, EcoRI; B, BamHI; X, Xba I; S, Sal I, Sc, Sac I.

**Fig. 2.** Southern blot analysis of human and transformant DNAs showing the first (A), second (B), and last (C) rearrangement as shown in Fig. 1 going from the left to the right of the map. DNA (10 μg) was digested with the enzymes indicated. The nick-translated probes used are also indicated (see the map in Fig. 1). Lanes: 1, NIH 3T3 DNA; 2, primary focus before agar selection; 3, same as in lane 2 but subcloned in agar; 4, Neo-selected secondary transformant Neo-2; 5, tertiary transformant derived from the transfection of the Neo-2 DNA; 6, FS4, human foreskin fibroblasts; 7, original KS tumor DNA used in the primary transfection (lanes 1 and 2 were exposed longer to enhance band intensity); 8, the A15-I tumor obtained in nude mice injected with the primary transformant shown in lane 3; 9, second tumor obtained in nude mice (A15-II); 10, secondary transformant F1A1 obtained by focus selection. Sizes are shown in kb.
The human DNA sequences present in the NIH 3T3 transformants contain a portion of the human c-fms gene. The human probe, which corresponds to the complete cDNA, recognizes in the mouse transformants the same extra bands hybridizing to the v-fms DNA 3' half probe. This confirms that only a 3' portion of the human c-fms sequences is present in our transformants.

Surprisingly, however, both v-fms and human c-fms cDNA probes failed to hybridize with any of the mRNA species recognized by our genomic probes in the NIH 3T3 transformants (data not shown); thus, the c-fms sequences do not appear to be transcribed.

**Biological Activity.** We transfected DNA from the individual recombinant phages shown in Fig. 1 into NIH 3T3 cells, but only the WI-1 phage was capable of producing foci alone, albeit at low efficiency, which was not increased by cotransfection with phage KS12. To map more precisely the transforming DNA sequences and to assess the relevance of the c-fms DNA, an 11-kb DNA fragment going from the left polylinker Sal I site of phage WI-1 rightward to the next Sal I site (Fig. 1) was subcloned into the Xho I site of the pCD SV40 expression vector (18) in both orientations with respect to the SV40 promoter, and the resulting plasmids were tested for biological activity. Both plasmids produced transformed foci on NIH 3T3 and rat F2408 cells with an efficiency comparable to that of a control plasmid (pTB-1; ref. 25) containing an activated ras gene (Table 1). Cells transformed by the pCD10 plasmids grew readily in agar medium. The same DNA fragment was also capable of producing transforming foci when inserted into the pGEM-3 bacterial vector, but with an efficiency lower by a factor of ~10.

We also inserted into both pCD and pGEM vectors a 6.6-kb DNA fragment that lacks the c-fms coding sequences and that goes from the left Sal I site of phage WI-2 to the same Sal I site used for the above-mentioned constructs (Fig. 1). The pCD6.6 constructs transformed both mouse and rat cells with high efficiency, similar to that of pTB-1 and of the pCD10 plasmids (Table 1), while the pGEM construct transformed with an efficiency lower by a factor of ~40. Therefore, the 6.6-kb fragment appears to contain all or most of the sequences encoding a transforming gene product(s) and also a transcriptional promoter because it can function in a plasmid vector devoid of mammalian transcriptional regulatory sequences.

**Table 1.** Transformation of mouse and rat fibroblasts with recombinant plasmid DNAs

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>DNA input, μg per culture</th>
<th>Foci per μg of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIH 3T3</td>
<td>Rat F2408</td>
</tr>
<tr>
<td>pCD(WII-1)I10A*</td>
<td>0.5</td>
<td>900</td>
</tr>
<tr>
<td>pCD(WII-1)I10B*</td>
<td>0.5</td>
<td>800</td>
</tr>
<tr>
<td>pGEM(WII-1)I10</td>
<td>0.5</td>
<td>120</td>
</tr>
<tr>
<td>pTB-1(ras)</td>
<td>0.2</td>
<td>800</td>
</tr>
<tr>
<td>pCD(WII-1)I26.6A*</td>
<td>1.0</td>
<td>2500</td>
</tr>
<tr>
<td>pCD(WII-1)I26.6B*</td>
<td>1.0</td>
<td>1400</td>
</tr>
<tr>
<td>pGEM(WII-1)I26.6</td>
<td>2.0</td>
<td>40</td>
</tr>
<tr>
<td>pTB-1</td>
<td>0.5</td>
<td>2500</td>
</tr>
</tbody>
</table>

Cells (~1 × 10^6 per dish) were transfected with the indicated amounts of plasmid DNA together with 20 μg of mouse carrier DNA. Each culture was then subdivided into five plates. Foci were counted 2-3 weeks after transfection. Note that the overall efficiency of transfection for NIH 3T3 was much higher in experiment II.

*A and B indicate the position of the SV40 promoter/enhancer element with respect to the polarity of transcription (going from left to the right in Fig. 1) of the inserted Sal I genomic fragments contained in the pCD expression vector. The "A" constructs have the SV40 promoter/enhancer in the 5' position, and the "B" constructs, in 3' position.
tory elements. Although the higher efficiency of transformation of the pCD plasmids is probably due to the presence of the SV40 enhancer, the relatively higher transforming ability of the pGEM10 plasmid with respect to the pGEM6.6 plasmid suggests that transcription regulatory elements may be missing in the plasmid with the shorter insert. Such elements could be contained in the c-fms region.

**Generation of the KS Oncogene.** The KS oncogene appears to be a previously unrecognized gene structure, apparently originating from DNA rearrangements involving the c-fms gene and two other unknown stretches of human DNA. Therefore, it becomes important to ascertain whether these rearrangements existed in the original tumor DNA or were generated during gene transfer (26-28). We used probes corresponding to the first, second, and fourth rearrangement detected in our cloned DNA sequences to determine whether the original KS necropsy DNA contained these rearrangements, and we could not detect them (Fig. 2A and data not shown). This suggests that these rearrangements had been generated during the first DNA transfection and faithfully transmitted thereafter. It has to be considered, however, that the original KS DNA had been extracted from three pooled skin lesions occurring in neighboring areas of the patient’s skin and that the proportion of the tumor cells in the lesion is not known and could be quite low, particularly in KS lesions that are often histologically mixed (3, 5). If the proportion of cells containing the “activated” rearranged oncogene in the pooled lesion had been <10%, we would be unlikely to detect the rearrangements in Southern blot hybridization with total DNA.

**DISCUSSION**

By transfection of NIH 3T3 cells with DNA from a KS necropsy, we have identified a human transforming gene that contains several DNA rearrangements and a portion of the human c-fms gene. These rearrangements are not detectable in the original tumor DNA, which also does not appear to contain rearranged c-fms sequences. Therefore, this oncogene must be provisionally considered to have been generated during transfection. We must emphasize that this conclusion could be wrong, since the initial tumor cell population from which the DNA was extracted could have been very heterogeneous and contain only a minority of cells having the rearranged “activated” oncogene.

The transfected human DNA sequences that we have recovered from our mouse transformants appear to contain four rearrangements with respect to normal human DNA. The first rearrangement from the left on the physical map in Fig. 1 is probably not important for the transforming activity since it is not present in some secondary or tertiary transformants and maps far away from the transforming region identified in the transfected sequences. The second and third rearrangements bracket the portion of the c-fms gene present in our cloned DNA. The latter of these rearrangements may contribute regulatory elements present within the c-fms genomic DNA that activate the transcription of sequences downstream of this region. The last rearrangement joins two unknown fragments of DNA, and its role in transformation is unclear at the moment.

An intriguing feature of the KS oncogene is the contribution of the c-fms sequences to its transforming activity. As described under Results, the c-fms sequences are not represented in stable mRNAs and are not essential for transforming activity. Furthermore, experiments of hybridization with probes corresponding to different fragments of the v-fms cDNA (21, 22) indicate that the 5'→3' polarity of the c-fms sequences is from right to left, in the opposite direction of the polarity of transcription of the two major species of mRNAs detected in our transformants (data not shown). Thus, we conclude that c-fms coding sequences are not part of the KS oncogene. The c-fms genomic sequences could however, contribute transcription regulatory elements, such as enhancers, as suggested by the reduced transforming ability of the pGEM6.6 with respect to the pGEM10 plasmids (Table 1).

The KS oncogene appears to direct the synthesis of at least two species of mRNAs, which are likely to represent transcripts of human DNA sequences that have been brought together and “activated” by rearrangements. It is likely, however, that sequences present in the shortest message may be sufficient for transformation, as cells transformed by both the pCD10 and pCD6.6 plasmids do not appear to synthesize a bona fide 3.5-kb messenger but only the 1.2-kb species and, in some cases, longer messengers of variable length (data not shown). However, this is only, at best, suggestive evidence because it is not known whether the two mRNAs are translated in the same reading frame.

Clearly much further work is necessary to characterize the KS oncogene, the nature and function of its products, and its possible involvement in human tumors. Partial sequencing of the cDNAs corresponding to the two mRNAs detected in transformants indicates that we have identified previously unrecognized transforming sequences. Their precise identification should be valuable to elucidate the multiple ways by which normal growth control can be subverted in cancer cells.

We thank Earl Nonon, Eva Deutsch, and Vera Levitska for their skilled assistance, and G. Teeber, F. G. Kern, M. Itttam, and A. M. Curatola for help and discussions. This investigation was supported by Grants CA37295 and CA42568 from the National Cancer Institute.