Activation of Transcription of the Melanoma Inducing Xmrk Oncogene by a GC Box Element*

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Melanoma formation in Xiphophorus is caused by overexpression of the Xmrk gene. The promoter region of the Xmrk oncogene differs strikingly from the corresponding proto-oncogenic sequences and was acquired in the course of a nonhomologous recombination with another gene locus, D. In order to identify regulatory elements leading to the strong transcriptional activation of Xmrk in melanoma tissue and to contribute to an understanding of the role the regulatory locus R might play in suppressing the tumor phenotype in wild-type Xiphophorus, we performed functional analysis of the Xmrk oncogene promoter. Transient transfections in melanoma and nonmelanoma cells revealed the existence of a potent positive regulatory element positioned close to the transcriptional start site. Contained within this promoter segment is a GC-rich sequence identical to the binding site described for human Sp1. In vitro binding studies and biochemical characterizations demonstrated the existence of GC-binding proteins in fish that share immunological properties with members of the human Sp family of transcription factors and appear to be involved in the high transcriptional activation of the Xmrk oncogene. Since the identified cis element is functional in both melanoma and nonmelanoma cells, additional silencer elements suppressing Xmrk expression in nonpigment cells must exist, thereby suggesting a negative regulatory function for the genetically defined R locus.

Molecular genetic analyses revealed that both versions of Xmrk, proto-oncogene and oncogene, are highly identical in their coding region but differ significantly in their promoter regions (11). This situation is explained by a nonhomologous recombination event between the Xmrk proto-oncogene and another gene locus (designated D) (12), giving rise to the oncogenic as an additional copy of Xmrk with altered 5′ sequences. This upstream region contains TATA- and CAAT-like sequences and could thus represent a “non-housekeeping gene” promoter (13) in contrast to the GC-rich sequences driving transcription of closely related receptor tyrosine kinases like the human epidermal growth factor receptor (14) or the rat HER2/neu gene (15). It is suggestive that these newly acquired upstream sequences account for the observed Xmrk overexpression in Xiphophorus hybrids and that the R locus might be involved in their transcriptional regulation. Analysis of the Xmrk oncogene promoter and its transcriptional control elements is therefore important to obtain insight into the mecha-

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1 The abbreviations used are: Xmrk, Xiphophorus melanoma receptor kinase; bp, base pair(s); PSSM, platy swordtail melanoma; CAT, chlor-amphencil acetyltransferase; HSV, herpes simplex virus; tk, thymi-dine kinase; CMV, cytomegalovirus; mut, mutant; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay.

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EXPERIMENTAL PROCEDURES

Enzymatic DNA Amplification of the Xmrk Oncogene Sd Allele Promoter—To obtain sequences of the Xmrk promoter upstream of the predicted breakpoint region (11), a PCR was performed on genomic DNA from Xiphophorus maculatus of the genotype SdStr/SdStr using a downstream primer from the Xmrk breakpoint region (DA 11) and an upstream primer (JA 8) derived from the D locus (12). PCR amplification was performed in a total volume of 50 μl with 200 ng of genomic DNA as template and 2.5 units of Taq polymerase. The buffer conditions were 100 mM Tris-Cl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% gelatin, 1% Triton X-100. After initial denaturation for 4 min at 92°C, amplification was performed for 35 cycles in a two-step PCR with 70°C as annealing/extension temperature. The primers used had the following sequences. DA 11, 5'-CTCCTTCTCCGGGTCTGTCGTGCAGCAGC-3'; JA 8, 5'-CTCGGATCCCTCAAGGCAGACTGG-3'.

The resulting 0.8-kilobase amplification product was specific for the Xmrk oncogene Sd allele. 3 To minimize the risk of cloning DNA sequences, DA 11, 5'-CTCCTTCTCCGGGTCTGTCGTGCAGCAGC-3' and JA 8, 5'-CTCGGATCCCTCAAGGCAGACTGG-3' were used.

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The embryonic epithelial cell line A2 (18) was transfected with XmrkTATACATI166, an 840-bp promoter fragment containing 107/20 was inserted into ptkTATACATII, and the lysate was centrifuged in a SS-34 rotor for 8 min at 40,000 rpm. The supernatant was passed over a PD-10 desalting column. Cellular debris was removed by centrifugation for 15 min at 14,000 rpm, and the supernatants were used for all following steps. The extract was aliquoted, frozen in liquid nitrogen, and stored at −80°C.

Electrophoretic Mobility Shift Assay (EMSA)—The oligonucleotides used as probes were annealed and labeled at the 5'-ends using T4 polynucleotide kinase and γ[32P]ATP (6000 Ci/mmol). The double-stranded probes were purified over a 15% polyacrylamide gel, eluted, precipitated with ethanol, and resuspended in TE buffer. Binding reactions were actions were performed in a final volume of 20 μl containing Xmrk promoter extract, 2 μg of poly(dI:dC)poly(dI:dC), 2 μg/ml bovine serum albumin, 90 mM KCl, and specific competitor DNA as indicated. The binding buffer consisted of 10 mM HEPES-KOH, pH 7.8, 2.5 mM EDTA, 5 mM spermine, 2% Ficoll 400, 6% glycerol, 1 mM diithiothreitol, and 0.5 mM PMSF. After 10 min incubation on ice, the cells were lysed by homogenization in a Dounce homogenizer with 12 strokes using a "B" pestle. 4 The addition of 10 × PBS to stabilize the nuclei, the lysate was centrifuged in a SS-34 rotor for 8 min at 4000 rpm. The nuclear pellet was resuspended in 3 packed cell volumes 50 mM Tris-Cl, pH 7.5, 10% sucrose, 420 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 20% glycerol, 1 mM diithiothreitol, 1 mM benzamidine, and 0.5 mM PMSF. The lysate was gently stirred for 1 h at 4°C and then centrifuged for 1 h at 40,000 rpm. The supernatant was passed over a PD-10 desalting column (Amersham Pharmacia Biotech) equilibrated with 20 mM HEPES-KOH, pH 7.8, 12.5 mM MgCl₂, 1 mM EDTA, 100 mM KCl, 0.1% Nonidet P-40, 20% glycerol, 1 mM diithiothreitol, 1 mM benzamidine, and 0.5 mM PMSF, and eluted in the same buffer. The extract was aliquoted, frozen in liquid nitrogen, and stored at −80°C.

Electrophoretic Mobility Shift Assay (EMSA)—The oligonucleotides used as probes were annealed and labeled at the 5'-ends using T4 polynucleotide kinase and γ[32P]ATP (6000 Ci/mmol). The double-stranded probes were purified over a 15% polyacrylamide gel, eluted, precipitated with ethanol, and resuspended in TE buffer. Binding reactions were actions were performed in a final volume of 20 μl containing Xmrk promoter extract, 2 μg of poly(dI:dC)poly(dI:dC), 2 μg/ml bovine serum albumin, 90 mM KCl, and specific competitor DNA as indicated. The binding buffer consisted of 10 mM HEPES-KOH, pH 7.8, 2.5 mM EDTA, 5 mM spermine, 2% Ficoll 400, 6% glycerol, 1 mM diithiothreitol, and 0.5 mM PMSF. After a 15-min preincubation step at 4°C, 20,000 cpm of labeled oligonucleotide was added, and incubation was continued for 15 min. For supershift assays 1 μl of each preimmune, anti-Sp1, or anti-Sp3 antiserum (26) was added to the binding reaction 10 min prior to loading of the gel. DNA-protein complexes were resolved on 10% polyacrylamide gels containing 5% glycerol and 0.25% Triton X-100.-bound complexes were visualized by autoradiography.

Copper-Phenanthroline Footprinting—According to Papavassiliou et al. (27) an upregulated EMSA reaction was performed, using a DNA fragment as probe labeled exclusively at one of its 5'-ends. Following electro-
phoresis the gel was immersed in 200 ml of 10 mM Tris-Cl, pH 8.0. After addition of 20 ml 2 mM 1,10-phenanthroline monohydrate, 0.45 mM CuSO_4, the chemical nuclease reaction was initiated by adding 20 ml of 58 mM 3-mercaptopropionic acid. The reaction was quenched after 7 min at 4 °C by adding 20 ml of 28 mM 2,9-dimethyl-1,10-phenanthroline monohydrate. After 2 min incubation the gel was rinsed four times in distilled water and subsequently electrophoresed on DE-81 membrane for 5 h at 500 mA in 0.5 × TBE. The membrane was autoradiographed overnight, and bands corresponding to free and bound probe were cut out. The membranes were washed twice with 100 μl of LS wash buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10 mM EDTA, pH 8.0) and then eluted twice with 100 μl of HS wash buffer (50 mM Tris-Cl, pH 8.0, 1 μM NaCl, 10 mM EDTA, pH 8.0) for recovery of DNA. Following sequential extractions with phenol/chloroform (1:1 (v/v)) and chloroform the DNA was precipitated with ethanol. After resuspension in 5 μl of loading buffer (50% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol), equal amounts (as determined by Cherenkov counting) were loaded as primary antibodies. After incubation with primary antibody overnight at 4°C and washing three times for 20 min at room temperature, the filters were incubated with horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature, the filters were incubated with horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature and washed again as above. Nonradioactive detection (enhanced chemiluminescence, Amersham Corp.) was performed according to the manufacturer's recommendations. To reprobe Western blots with different antibodies, the membrane was incubated in STRIP buffer (62.5 mM Tris-Cl, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol) for 1 h at 50 °C. To remove any remaining SDS, filters were washed three times in PBS and then blocked again for 1 h with NETG.

RESULTS

Identification of Regulatory Elements within the Xmrk Promoter Proximal Region—Sequences further upstream of the previously isolated promoter region of the Xmrk oncogene (11) were obtained by PCR using primers deduced from the D locus (12) and the Xmrk coding region. This segment containing 675 bp upstream of the transcriptional start site was inserted into a CAT reporter gene vector to yield XmrkCAT −675/+34. Transient transfection of this promoter construct into two different Xiphophorus cell lines of melanoma (PSM) and nonmelanoma (A2) origin revealed similar high activity in both cell types (Fig. 1). The seemingly different transcriptional activity of Xmrk −675/+34 observed upon comparison with a HSV tk promoter construct was only due to a differential transcriptional potential of the viral promoter in the two cell lines, which was demonstrated using a construct containing only the tk-derived TATA box as a reference. Thus, the existence of one or several strong, positive regulatory element(s) within the Xmrk −675/+34 region has to be proposed, driving transcription of the Xmrk oncogene in a non-tissue-specific manner.

Gene transfer of the Xmrk −675/+34 CAT fusion into early embryos of medaka fish (Oryzias latipes), a genus closely related to Xiphophorus, revealed that the investigated promoter fragment was not only active in tissue culture cells but was also functional in vivo in a whole animal system (Fig. 2). CAT activity observed in embryos 3 days after injection with Xmrk −675/+34 CAT was on average about 50% of CAT expression driven by one of the strongest enhancer-promoter combinations in this in vivo assay. CMV-tk, indicating the high transcriptional activity of the Xmrk regulatory sequences. A promoter-less CAT construct sharing the plasmid backbone with Xmrk −675/+34 CAT led to expression at background level comparable with that of noninjected embryos.

To determine the position of the regulatory elements responsible for this high level activation, the reporter gene activity of a set of 5′ deletions spanning the −675/+34 segment of the Xmrk upstream region was quantitated in the melanoma cell line PSM. Whereas deletion to −194 led to a reduction in reporter activity to about 60% of −675/+34 (Fig. 3A), an even larger decrease in promoter activity to approximately 20% of −675/+34 was observed in a construct lacking sequences upstream of −49. These results indicate the presence of probably two positive cis regulatory elements positioned in the promoter region downstream of −277 and −125, respectively. For a more precise localization of the regulatory elements various Xmrk promoter fragments were then fused to the tk promoter and
tested for their transcriptional activation potential (Fig. 3B).
Since fragments −277/+34 rev and −194/−20 did not differ significantly regarding their CAT expression, obviously no enhancer element was detectable in this assay within the region between −277 and −194. However, a fragment containing the region between −67 and −20 proved to be sufficient to activate a heterologous promoter to a similar level as all longer Xmrk fragments demonstrating the presence of functional activating elements within these 48 bp.

**DNA-Protein Interactions with Xmrk −67/−20**—To test whether the in vivo observed positive regulatory activity within −67 and −20 was also reflected by in vitro DNA-protein interactions, EMSA was performed using an 88-bp HindIII/BamHI fragment of Xmrk CAT −67/−20 as probe and nuclear extract of PSM cells (Fig. 4A). One major complex was formed with this fragment. To precisely localize the position of the complex we performed copper-phenanthroline footprint analysis applying the same conditions as in the previous EMSA. The complex produces one clear footprint positioned between −50 and −41 with a hypersensitive site at position −46 (Fig. 4B). Interestingly, the protected region coincides with a GC-rich region within positions −48 and −41 of the Xmrk promoter that has the sequence 5′-CCGGCCCC-3′, thus resembling the previously determined Sp1 consensus binding motif (29). These data suggest that the complex is formed over the Sp1 consensus within −67/−20, implying the possible involvement of DNA-binding proteins related to Sp1 in complex formation.

To test further whether Sp1-like proteins were involved in complex formation, EMSA was performed using an oligonucleotide as probe, which was derived from the Xmrk sequence between −67 and −32 (Xmrk −67/−32) (Fig. 5A), in order to avoid undesired side effects from the endogenous Xmrk TATA box positioned within residues −34 to −27. High resolution gel analysis of the DNA-protein complex revealed two bands of slightly different mobility (C, C′) (Fig. 5B). Both complexes proved to be specific since unlabeled Xmrk −67/−32 inhibited complex formation, whereas an oligonucleotide mutated in two positions within the GC box (Xmrk −67/−32 mut) did not, suggesting the involvement of Sp1-related transcription factors in the complex. This observation was confirmed by using an Sp1 consensus oligonucleotide as competitor (30) sharing only the Sp1 core sequence with Xmrk −67/−32, which also inhibi-
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FIG. 5. Detection of GC-binding activity in PSM nuclear extracts. A, upper strand sequences of oligonucleotides used in EMSAs presented in B and C. Substituted bases in Xmrk -67/-32 are indicated by asterisks. The GC-rich sequence shared between Xmrk -67/-32 and Sp1 consensus oligonucleotide (Sp1) is boxed. B, specificity of protein binding to Xmrk -67/-32. PSM nuclear extracts were incubated with radiolabeled Xmrk -67/-32 in the absence (-) or presence of increasing amounts (20- and 200-fold molar excess) of unlabeled double-stranded competitors Xmrk -67/-32 (wt), Xmrk -67/-32 mut (mut), Sp1, and an unrelated oligonucleotide comprising an AP1 binding site (AP1), respectively. The two complexes forming over Xmrk -67/-32 (C, C') are indicated by arrows; the control lane contains no protein in the EMSA reaction. C, protein binding to wild-type and mutated Xmrk GC sequences. Radiolabeled oligonucleotides containing the Xmrk -67/-32 region (wt) or the corresponding mutant (mut) altered within the GC box were used in an EMSA with PSM nuclear extracts.

Recent studies indicate that the zinc finger protein Sp1 is one of several members of a differentially expressed gene family (32, 33). In order to determine which of their corresponding fish homologues might bind to the Xmrk oncogene promoter, we performed supershift analyses with different antibodies raised against human Sp proteins. Whereas addition of a polyclonal anti-Sp1 antiserum as well as an antibody raised against residues 520–538 of the human Sp1 protein (data not shown) left both complexes unaltered, addition of an anti-Sp3 antiserum led to loss of complex C and a strong reduction of C', indicating the presence of a fish Sp3 homologue in the complexes (Fig. 6). Addition of an antiserum against human Sp4, a nuclear factor with a very limited expression pattern (32), as well as preimmune serum as control also showed no effect on complex formation (data not shown).

Sp-related Proteins in Xiphophorus—In various studies Sp1 has been reported to be ubiquitously expressed (29), and therefore its lack of detectability in the supershift assay was unexpected. The inability of both anti-Sp1 antiserum to reduce or supershift complexes C and C' could either result from the absence of Sp1 in the nuclear extracts or a lack of cross-reactivity of the antibodies toward fish Sp1 under EMSA conditions. To test this we conducted Western analyses of nuclear extracts prepared from PSM cells using different antibodies for detection (Fig. 7). An antibody raised against a peptide outside the zinc finger region of human Sp1 (aSp1(PEP2)), which identifies the human Sp1 95- and 106-kDa polypeptide species, recognized one major protein of about 80 kDa in melanoma cell nuclear extracts. A polyclonal serum against full-length Sp1 (aSp1) also detected a protein of approximately 80 kDa and in addition species of 85 and 75 kDa molecular mass. Immunodetection using anti-Sp3 antiserum also identified a prominent band of about 85 kDa. No such bands were observed using preimmune sera in control experiments. These results demonstrate the existence of fish homologues of human Sp1 and Sp3 in PSM cells. Hence, it seems likely that the inability of anti-Sp1 antibodies to supershift the complex formed over Xmrk -67/-32 was rather due to a lack of cross-reactivity under EMSA conditions than to the general absence of Sp1 in the fish cells.
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**DISCUSSION**

Overproduction of the Xmrk-encoded receptor tyrosine kinase has been shown to be responsible for melanoma formation in *Xiphophorus* hybrids (7, 11). Resulting from high steady state levels of the corresponding mRNA, this elevated expression is most likely due to a transcriptional deregulation of the Xmrk promoter in tumor cells. In order to contribute to an understanding of the mechanisms leading to tumor formation in *Xiphophorus*, it was important to identify regulatory elements that account for this high promoter activity. Preliminary experiments had revealed that a fragment comprising 277 bp upstream of the transcriptional start site of the Xmrk oncogene was able to drive the expression of a reporter gene (11). In this report we have isolated sequences further upstream of the originally described promoter and identified a potent cis element and its corresponding transacting factors enhancing transcription of the Xmrk oncogene. An Xmrk promoter fragment spanning the region −675/+34 was shown to be highly active in two different *Xiphophorus* cell lines of melanoma and nonmelanoma origin. In addition, the analyzed upstream sequences proved not only to be functional in tissue culture cells but also in fish embryos after transient gene transfer. Comparison with CMV tkCAT demonstrated the high level activation potential of the Xmrk promoter in vivo in a developing embryo. The variability of CAT activity observed in individual embryos could be explained by the mosaic distribution of the injected plasmid (10) leading to CAT expression only in a subset of tissues exhibiting responsiveness to the Xmrk promoter.

Whereas deletion analysis suggested the presence of two distinct positive regulatory elements downstream of positions −277 and −125, respectively, subsequent evaluation of various promoter fragments in a heterologous promoter context revealed the existence of only one potent positive regulatory element. The failure to detect enhancer activity within the more distal region in this type of analysis could be due to the requirement for an authentic Xmrk promoter context of this putative regulatory element to function. Such specific enhancer-promoter interactions have been described for several other genes and may represent a mechanism ensuring transcriptional specificity (34, 35). Within the proximal region, however, a 48-bp segment adjacent to the Xmrk TATA box proved to be able to confer strong transcriptional activity to a heterologous promoter.

By copper-orthophenanthroline footprinting analysis the DNA-protein complex formed over this region was shown to be positioned over a core sequence, 5'-CCGCGCCC-3', that is identical to the binding motif described for the zinc finger protein Sp1 (29). Mobility shift assays substantiated the finding that GC-binding proteins interacting specifically with the Xmrk GC box are present in *Xiphophorus* nuclear extracts. The competition observed between the Xmrk-derived binding site and the Sp1 consensus oligonucleotide, which are unrelated outside the core region, clearly demonstrates the crucial role of the GC box for the protein-DNA interaction. This is supported by the complete loss of protein binding upon mutation of two base pairs within the core consensus. In addition to the members of the Sp protein family (32, 33), other GC box binding proteins have been identified. Whereas the BTE binding factors seem to recognize a variant sequence motif deviating from the Sp1 consensus site analyzed here (36–38), the only other proteins described so far binding to this consensus are the yeast MIG1 repressor (39) and its fungal homologue CRE (40). MIG1 interacts specifically with the pentamucleotide motif 5'-GGG-3'; however, it requires flanking AT-rich sequences adopting a particular geometry for high affinity binding (41), making it unlikely that a factor of this nature binds to the Xmrk GC box.

Supporting evidence for the identity of the GC box binding factors in PSM cells is provided by the Western blot experiments indicating the presence of proteins that share structural features with members of the mammalian Sp family of transcription factors even outside the highly conserved zinc finger region. Consistent with these findings are supershift assays in which addition of an anti-Sp3 antiserum led to reduced formation of the respective DNA-protein complexes, indicating the involvement of different forms of a fish Sp3 homologue in complexes C and C', which is in accordance with observations made in mammalian cells (26). Anti-Sp1 antiserum was, in contrast, not able to exhibit an effect in this type of assay, although Sp1-related proteins are present in *Xiphophorus*. It is conceivable, however, that an antibody may only under certain conditions be able to cross-react with its corresponding

**FIG. 8.** Transcriptional activity of wild-type and mutant Xmrk GC boxes. PSM cells were transfected with CAT reporter plasmids under the control of the tk-derived TATA box fused to wild-type and mutant (mut) Xmrk promoter fragments in either forward (F) or reverse (R) orientation or an Sp1 consensus oligonucleotide as indicated (for sequences see Fig. 5A). CAT activities are normalized to the values obtained with a CAT construct containing the minimal promoter alone and are the average of at least four independent experiments with error bars representing standard errors of relative CAT activities (Mann-Whitney U test; *, p < 0.05).

Functional Analysis of the GC Box within Xmrk −67/−32—To determine whether the GC box constitutes an important functional cis element within the Xmrk promoter, the region between −67 and −32 was analyzed in more detail in a set of transient transfections in PSM cells (Fig. 8). Oligonucleotide Xmrk −67/−32 and its corresponding mutant carrying two nucleotide exchanges within the GC box (Xmrk −67/−32 mut) were inserted directly upstream of the tk TATA box and evaluated functionally in this heterologous promoter context. To avoid interference with the endogenous Sp1 site within the HSV tk promoter, the tk-derived TATA box was used. Xmrk −67/−20 and Xmrk −67/−32 in either orientation stimulated CAT activity to comparable levels, demonstrating that these sequences are sufficient to enhance reporter gene expression. Mutation of two base pairs within the GC box (Xmrk −67/−32 mut) resulted in a complete loss of activity. In accordance with these findings an isolated Sp1 site (30) sharing only an 8-bp core sequence with Xmrk −67/−32 (Fig. 5A) was able to drive CAT expression to a level which did not differ significantly from the Xmrk promoter fragment. These results clearly indicate that the GC box is the functional element within Xmrk −67/−32 and therefore critical for the activity of the Xmrk oncogene promoter.
Sp1 proteins are nearly identical to human Sp1 in their com-
from rat (37, 42), and several distant relatives have been isolated
of human Sp proteins have only been described for mouse and
mrk contained within the X

and that at least one of them is able to bind to the GC box

in the supershift experiments was rather due to its failure to
deletions. Based upon the observation that, when compared
thissequencewithintheXmrk
gene have been characterized.

The notion that high transcriptional activity of the Xmrk
promoter accounts for the overexpression of the Xmrk oncogene
in melanoma tissue is strongly supported by the presence of a
potent positive regulatory element identified within the Xmrk

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Transcriptional Activation of the Xmrk Oncogene by a GC Box
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Page 135, Fig. 5: The reproduction of this figure was not satisfactory. A better version is shown below.

A

B

C

FIG. 5

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