The Oncogene qin Codes for a Transcriptional Repressor

Jian Li,2 Hwai Wen Chang, Eseng Lai, Elizabeth J. Parker, and Peter K. Vogt

Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037 [J. L., H. W. C., P. K. V.]; Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [E. L.]; and Department of Cell and Molecular Biology, University of St. Andrews, St. Andrews, United Kingdom [E. J. P.]

Abstract

The retroviral oncogene qin codes for a protein that belongs to the winged helix family of transcriptional regulators. The Qin protein is localized in the nucleus and binds to the same DNA consensus sequence as rat brain factor 1 (BF-1). Cellular Qin shows greater affinity to DNA than does viral Qin. Alone or fused to the DNA-binding domain of the yeast GAL4 protein, both Qin proteins act as transcriptional repressors. The major transcriptional repression domain maps to the region of amino acids 252–395 of viral Qin.

Introduction

The oncogene qin was isolated from the genome of avian sarcoma virus 31, a highly oncogenic retrovirus found in a spontaneous connective tissue tumor of an adult chicken (1). Overexpression of the v-qin3 gene leads to transformation of CEFs in culture and causes fibrosarcomas in young chickens. Qin codes for a protein of the winged helix family of transcription factors (2, 3). A prototype member of this family is the Drosophila gene forkhead (fkh), a region-specific homeotic gene that promotes terminal development in the early embryo (4). Other important representatives are the liver-enriched transcription factors HNF3α, 3β, and 3γ, which also show exquisitely tissue-specific expression during embryogenesis (5, 6). Homozygous null mutations of HNF3β in mice lack organized node and notochord formation and cause early embryonal lethality (7, 8).

Materials and Methods

Plasmid Construction. The PCR-amplified fragments of chicken c-qin from nt 1–1521 and of v-qin as shown in Fig. 4 were cloned individually into the EcoRI-BamHI sites of the GEX-2T vector (Pharmacia). The chimeric vectors GST-CV and GST-VC were constructed by exchanging BamHI-SmaI fragments of GST-v-Qin and GST-c-Qin. PCR-mediated in vitro mutagenesis was carried out to construct the GST-Vm14 mutant. Using two oligonucleotides corresponding to nt 715–735 of v-qin, with mismatch mutations and primers derived from nt 1–18 and 1162–1188, the amino acid substitution (Asp→Gly) was generated, and a HindIII restriction site was introduced for cloning. These proteins were expressed in Escherichia coli VS 20, 186 cells and purified from the crude bacterial extract using glutathione agarose beads. The quantity of proteins used in DNA-binding assays was estimated by protein assay (Bio-Rad) and Western blot analysis. GAL4-Qin chimeras were constructed with plasmid pGAL0. This plasmid codes for the amino terminus of the yeast transcriptional activator GAL4 (aa 1–147) and allows in-frame cloning of DNA fragments to be expressed as GAL4 fusion proteins. All chimeras (pGly, pGli, pG84, pG19, and pGC) were constructed by PCR amplification of nt 1–1395, 1–141, 142–395, and 1–251 of v-Qin, and 1–451 of c-Qin (Fig. 7). Primers designed for PCR included EcoRI (NH2-terminal) and Xhol (COOH-terminal) restriction sites allowing in-frame cloning. Similarly, full-length c-qin or v-qin was inserted into the vector pRC/RSV (Invitrogen) to yield pRC/RSV-cQ and pRC/RSV-vQ. DNA fragments derived from PCR amplification were sequenced using oligonucleotide primers internal to the qin coding sequence or T3 and T7 primers. For immunofluorescence c-qin and v-qin were cloned and expressed in the retroviral vector RCAS (RCAS-c-qin and RCAS-v-qin) following published techniques (13).

Electrophoretic Mobility Shift Assays. Annealed oligonucleotides were labeled with Klenow polymerase in the presence of [32P]dCTP and purified by gel electrophoresis. GST proteins were incubated with 20,000 cpm labeled DNA probe and 3 μg of poly (dl·dC) in binding buffer [50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 20% glycerol] for 20 min at room temperature in 20 μl final volume. DNA-protein complexes were resolved on a 6% polyacrylamide gel in 0.5 × TBE buffer (45 mM Tris-borate-1 mM EDTA (pH 8.0)). The gels were dried and autoradiographed at −70°C.

Transient Transfection Assays. CEFs were plated in DMEM with 10% FCS at 0.5 × 106 cells/35-mm diameter well of 6-well tissue culture plates 24 h before transfection. Transfection with reporter and Qin expression plasmids followed the calcium phosphate technique (14), and cells were harvested 48 h after transfection. Luciferase or CAT activities in cell extracts containing equal amounts of protein were determined with a luminometer or phosphorimeter after TLC (14).

Antibodies and Immunofluorescence. The GST-c-Qin protein was purified by glutathione affinity chromatography. After glutathione elution, the GST portion was cleaved from Qin with thrombin, and Qin was gel purified. A rabbit was immunized with 3 injections of 500 μg c-Qin. An affinity-purified rabbit serum against a peptide extending from aa 142 to 148 of v-Qin was also used for immunofluorescence (15). The method for immunofluorescence staining has been described previously (16).

Results

Qin Is a Nuclear Protein That Binds to DNA with Similar Specificity as BF-1. Nuclear localization of Qin was demonstrated by immunofluorescence with an antipeptide serum directed against a hydrophilic region near the 5′ end of the DNA-binding domain of the Qin protein (Fig. 1). This pattern of nuclear staining was observed in cells transfected with the RCAS vector expressing either c-Qin or v-Qin alone, but not with vector controls. Nuclear localization of Qin could also be demonstrated with a rabbit serum against full length
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Fig. 1. Subcellular localization of the Qin protein. Immunofluorescent staining of chicken embryo fibroblasts infected with RCAS vector alone (A), RCAS-c-Qin (B), and RCAS-v-Qin (C) using a Qin specific antiserum raised against a peptide of aa 132-148 of the v-Qin protein. The antiserum also recognizes c-Qin. X250.

c-Qin (data not shown). The presence of the winged helix motif in Qin and the nuclear localization of Qin proteins suggested that qin may code for a DNA-binding protein. Electrophoretic mobility shift assays were, therefore, performed with GST-Qin fusion proteins. DNA-protein complexes were detected in assays of GST-c-Qin protein and two oligonucleotides (Fig. 2), one derived from a region named B2 in the HNF1α promoter (17), and the second, a PCR-selected oligonucleotide that binds optimally to BF-1 (18). These complexes were competed by excess unlabeled oligonucleotides and were supershifted by Qin-specific antibodies. B2 and BF-1 binding sites share the consensus sequence AATGTAAACA. Similar to BF-1, the Qin protein binds to the BF-1 site with stronger affinity than to the B2 site. Binding of Qin to a third site, the HNF3 target from the transthyretin gene promoter was barely detectable (data not shown). These results, together with sequence homology and telencephalon-specific expression, are in accordance with the proposal that qin is the avian homologue of the BF-1 gene.

v-Qin carries several mutations that distinguish it from its progenitor chicken c-Qin (15). These mutations may have an effect on Qin function. Comparing equal amounts of GST-Qin fusion proteins by electrophoretic mobility shift assays with the BF-1 and the B2 binding sequences, c-Qin showed significantly higher DNA-binding activity than v-Qin (Fig. 3A). The v-Qin complex with DNA was detected only when large amounts of GST fusion protein were used. The intensity of the shifted band with v-Qin was also reduced by lower concentrations of specific unlabeled oligonucleotide competitor than was the intensity of the DNA-cQin band. Two chimeric constructs of v-qin and c-qin were generated by using the SmaI site located at the middle of these genes (nt 794-799 of c-qin). Comparison of the two chimeric proteins in electrophoretic mobility shift assays suggests that the reduced DNA-binding activity is linked to an aa substitution (Gly→Asp) within the winged helix motif of v-Qin (Fig. 3B). This conclusion was further confirmed by using a v-Qin mutant, GST-Vm14, in which aspartic acid (residue 241) was mutated to glycine, which occupies this position in c-Qin. GST-Vm14 binds to the BF-1 site with an affinity that is similar to that of c-Qin.

The region of Qin required for DNA binding was defined by deletion analysis (Fig. 4). Deletions (3') of v-qin were constructed with a 5' sense primer corresponding to nt 1-18 in combination with various 3' antisense primers. Similarly, 5' deletions were generated with a 3' antisense primer derived from nt 1162-1188 and various 5' sense primers. Analysis of these mutants by electrophoretic mobility shift assays revealed that removal of 165 aa from the COOH-terminal end of Qin (deletion C2) or the truncation of the 186 NH2-terminal end aa (deletion N4) effectively abolishes DNA-binding activity. The region eliminated in both C2 and N4 deletions includes the W1, W2, and H3 structures of the winged helix motif (19). Deletions of NH2- or COOH-terminal sequences outside the winged helix motif did not affect DNA binding.

Qin Acts as a Transcriptional Repressor. Possible transcriptional regulatory activities of Qin were examined with transient trans-
Fig. 3. Qin proteins bind to DNA with different affinities. An oligonucleotide probe with one copy of the 22-bp containing BF-1-binding sequence was used in these assays. A, different amounts (0.1, 0.3, and 1 μg) of GST-c-Qin and GST-v-Qin proteins bind to the labeled probe (right part of Fig. 3A). One μg of the GST-Qin protein is competed with increasing amounts (10, 100, and 500 ng) of unlabeled BF-1-binding oligonucleotide (left part of Fig. 3A). B, DNA-binding activities of GST-VC, GST-CV chimeric proteins (1 μg), and GST-VM14 mutant as well as GST-VQin (0.5 μg). C, construction of Qin mutants. ■, eight amino acid leader sequence; ◇, homologous region to HNF3 transactivation domain; □, histidine-proline-rich region; ◇, zipper like motif; □, winged helix motif.

Fig. 4. DNA-binding activities of Qin deletion mutants. PCR-amplified fragments of v-qin were cloned into the pGEX-2T vector and generated GST-Qin fusion proteins containing the full-length or truncated versions of the v-Qin protein. After glutathione elution, equal amounts of protein (1 μg) from each construct were incubated with the labeled BF-1-binding sequence for electrophoretic mobility shift assays. Structural features of the Qin protein are shown as in Fig. 3.
expression is barely detectable, primary brain cultures from chicken embryos express Qin. The results of transient transfections into these neural cell cultures were the same as those obtained with CEFs (data not shown).

To determine whether Qin also functions as a transcriptional repressor when it is transferred to a heterologous DNA-binding domain, GAL4-Qin chimeric proteins were constructed (20). The full-length c-Qin and v-Qin cDNAs were cloned into the GAL4 expression vector pGAL0. The expressed fusion proteins contain the yeast GAL4 DNA-binding domain at their NH2 terminus. When the GAL4-Qin constructs were cotransfected into CEFs with the reporter plasmid pGAL4-TK-CAT, which carries the CAT gene under the control of the TK promoter with five GAL4-binding sites, strong repression of CAT activity was observed (Fig. 6A). Transcription was also reduced with a control reporter plasmid pTK-CAT, which does not contain the GAL4 binding site. The repression observed with GAL4-Qin appears, therefore, to be not completely dependent on binding of the fusion proteins to the GAL4 site. As a further control, the pGAL0 expression vector alone, lacking qin sequences, was cotransfected with both reporters. It was surprising that a small reduction of CAT activity was again detected, even when the pTK-CAT reporter was used. This result suggests that part of the binding site-independent repression seen in the GAL4-Qin/pTK-CAT cotransfection may be contributed by the pGAL0 plasmid. However, in the GAL4-Qin/pGAL4-TK-CAT combination, only a minor portion of the observed repression would be due to the nonspecific action of the pGAL0 sequences. We next mapped the domains of the Qin protein required for transcriptional repression (Fig. 6B). Three deletion mutants of v-Qin were fused to

![Diagram A](image1)

![Diagram B](image2)

Fig. 6. A, transcriptional repression mediated by a GAL4-Qin fusion protein. a, diagram of two CAT reporter constructs used in transient transfection assays. b, each reporter plasmid (2 μg) was cotransfected with the indicated amount of pGAL0 vector or a GAL4-vQin chimera plasmid, pGVQ into CEF for CAT assays. b, deletion analysis of the repression domain of the Qin protein. The full-length and deleted derivatives of v-qin were cloned into the pGAL0 vector and cotransfected with the pGAL.TK.CAT reporter plasmid into CEF for CAT assays. A GAL4-Qin construct containing the complete coding region of chicken c-qin, pGCQ, was also included. The structures of the Qin proteins are depicted in Fig. 3. I, GAL4 (aa 1–14); II, COOH terminus of Qin.

the GAL4 DNA-binding domain and assayed. The pGAL0 plasmid was used as a background control. The major transcriptional repression domain was located at the COOH terminus of v-Qin, in the region between aa 252 and 395. A RCAS construct containing a deletion of this region also failed to induce oncogenic transformation of CEF, suggesting that the repressor activity of Qin may be required for oncogenicity.4

Discussion

The DNA target sequences of Qin include the B2 site from the promoter of the HNF1α gene and the concensus binding sequence for BF-1. Qin binds strongly to the BF-1 site, but interacts only weakly with B2. The specificity of this DNA target, the sequence homology to BF-1, and the brain-specific expression pattern shared with BF-1 are all in agreement with the conclusion that Qin is the avian homologue of BF-1. v-Qin and c-Qin differ in their affinity to the BF-1 site. The two proteins diverge structurally at several sites: v-Qin has an 8-aa cell-derived NH2-terminal leader peptide. It carries two single nonconservative aa substitutions, one in the DNA-binding region and the other in a region that is homologous to one of the transactivation domains of HNF3, and it has suffered a 71-aa COOH-terminal dele-

4 Unpublished data.
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Mapping and mutagenesis data link the aa substitution in the DNA-binding domain of v-Qin to reduced affinity for the BF-1 site. c-Qin and v-Qin also differ in oncogenicity. v-Qin rapidly causes tumors in young chickens within a short latent period and a high efficiency, whereas c-Qin is only marginally if at all oncogenic. Experiments are in progress to test the hypothesis that the altered DNA-binding properties of v-Qin are related to oncogenicity either by changing the regulation of target genes shared with c-Qin or by allowing interaction with targets that are not affected by c-Qin.

In the cell systems tested, the Qin proteins act as transcriptional repressors. The major repression domain maps to the COOH-terminal third of Qin. Three possible mechanisms exist for transcriptional repression by Qin: (a) squelching (i.e., Qin would bind to and neutralize a positive transcriptional regulator); (b) competition with a positive regulator for the same DNA target; and (c) direct repression by binding to the DNA target and issuing a negative regulatory signal. The available data do not distinguish between these possibilities. However, the Qin sequence contains an oligo-alanine stretch similar to known Drosophila krüppel (21), even-skipped (22), and engrailed (23), which act by direct repression. The transcriptional repressor activity of Qin was unexpected because Qin contains NH2-terminal sequences that are homologous to one of the transactivation domains of HNF3 (12). It is conceivable that Qin is bifunctional; it may act as either repressor or activator of transcription, depending on cellular context.

Transcriptional repressors play essential roles during cell differentiation and development. One early acting gene of Drosophila, tramtrack, suppresses inappropriate transcription of several pair-rule genes in the process of segmentation (24). An example in mammals is the neuron-restrictive silencer factor that binds to a specific DNA sequence and represses neuronal gene transcription in nonneuronal cells (25). Qin may share general functional properties with these genes and serve as a negative regulator determining regional differentiation of the neural tube (10). A null mutation of the homologous BF-1 causes embryonic death and reduction in the size of the cerebral hemispheres (11). The BF-1 gene may play an important role in the morphogenesis of the telencephalon, possibly by regulating the rate of neuroepithelial cell proliferation and the onset of neuronal differentiation. Sequences related to Qin/BF-1 outside the widely shared winged helix domain have been found not only in all vertebrates tested but also in yeast (26, 27). This high evolutionary conservation of Qin-specific domains suggests important regulatory functions that may go back to early unicellular eukaryotes.

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