A Rearranged JunD Transforms Chicken Embryo Fibroblasts

Markus Hartl2 and Peter K. Vogt
Department of Microbiology and Norris Cancer Center, University of Southern California School of Medicine, Los Angeles, California 90033

Abstract
A complementary DNA clone synthesized from the chicken junD mRNA, containing 5′- and 3′-untranslated sequences, was inserted in the retroviral expression vector RCAS to yield the construct JD. A second RCAS construct (DDDD) contained only the coding domains of JunD. DDDD did not transform upon primary transfection, but JD produced small numbers of transformed cell foci in chicken embryo fibroblast cultures. The virus recovered from these foci, JDV, was moderately transforming for chicken fibroblasts and weakly oncogenic in the animal. Its genome was rearranged, showing evidence for two recombination events. The first crossover was located between 5′-untranslated and coding sequences of junD and incorporated part of the 5′-untranslated region into an open reading frame. The second crossover occurred between junD and gag. The two crossovers generate a single open reading frame of 2064 nucleotides that encodes an 85 kilodalton protein in which sequences in the amino-terminal region of JunD are duplicated. This gag junD reading frame was recloned and then reconstituted into a replication-defective but transformation-competent retrovirus, indicating that the Gag-JunD fusion protein is the effector of transformation. A construct containing this rearranged coding sequence of JunD in Rc/RSV transactivated the collagenase promoter in chicken cells. Southern blot analysis of several independently isolated JunD transformants and deletion analysis of JDV indicated that duplication of a domain in the amino-terminal region of JunD is crucial for transformation and transactivation.

Introduction
The jun gene is a member of a multigene family. jun itself codes for a major component of the AP-1 transcription factor that plays an important role in the control of gene expression, converting short-term incoming growth signals into long term programmed cellular responses (reviewed in Refs. 1–3). The Jun protein also functions as modulator of other transcription factors (4–7). Besides jun, vertebrate genomes contain two closely related genes, junB and junD (8, 9). The functions of these jun-related genes are not well understood. junB is, like jun, an “immediate early” gene whose transcription is turned on transiently by mitotic signals (8). Under some conditions, it can interfere with the activities of jun (10, 11). The expression of junD is not responsive to growth signals; junD is constitutively expressed at low levels in many cell types (9, 12, 13).

We have cloned and sequenced the junD gene of the chicken (13). The JunD protein, overexpressed in CEFs, fails to transactivate the collagenase promoter, inhibits transactivation by Jun, and does not induce oncogenic transformation (13–15). However, several chimeric constructs combining sequences from Jun and JunD are oncogenic, suggesting that JunD contains domains that support transforming activity (14, 15). Here, we describe a rearranged JunD that is fused to retroviral Gag sequences, has acquired an internal duplication, and with it has gained the capacity to transform chicken embryo fibroblasts.

Results
A Transforming Virus Emerges from JunD-transfected CEFs. In tests for transforming activity, JunD protein was expressed using two RACS constructs: one, termed DDDD (15), contained only the JunD coding sequences; the other, referred to as JD, represented the cDNA clone of chicken junD including 5′- and 3′-untranslated regions (Fig. 1). The RCAS vector carrying the DDDD insert did not induce foci of transformed CEFs under agar overlay after primary transfection (Table 1). However, if the transfected cells were transferred repeatedly, the entire culture eventually became transformed. Presumably, the original DDDD insert is nontransforming, but replication of the RCAS DDDD retrovirus generates transforming variants. In cultures transfected with the second construct, RCAS-JD, single rare transformed foci were reproducibly observed (Table 1). These foci could have arisen from an innate transforming potential of JD, or they could represent clonal genetic variants. Assuming the latter possibility, we chose to investigate the JD-induced transformation.

Foci of JD-transformed cells were picked and plated on CEF feeders. These cultures released low to moderate titers of a transforming virus (~10⁴ focus-forming units/ml). The virus was injected into the wing web of newly

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3 The abbreviations used are: CEF, chicken embryo fibroblast; cDNA, complementary DNA; kb, kilobase(s); LTR, long terminal repeat; kD, kilodalton(s); COL, collagenase promoter; CAT, chloramphenicol acetyltransferase; SSC, standard saline citrate; SDS, sodium dodecyl sulfate.
Table 1   Transformation of CEFs expressing junD from the RCAS vector

<table>
<thead>
<tr>
<th>Transfected construct</th>
<th>Foci/500 ng DNA</th>
<th>Protein expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCAS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RCAS VC-3</td>
<td>150</td>
<td>4+</td>
</tr>
<tr>
<td>RCAS DDDD</td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td>RCAS JD</td>
<td>0-1</td>
<td>1+</td>
</tr>
<tr>
<td>RCAS JDV</td>
<td>5</td>
<td>2+</td>
</tr>
<tr>
<td>RCAS JDV+RCAS</td>
<td>58</td>
<td>2+</td>
</tr>
<tr>
<td>RCAS JDV-N</td>
<td>0</td>
<td>2+</td>
</tr>
<tr>
<td>RCAS JDV-N+RCAS</td>
<td>0</td>
<td>2+</td>
</tr>
</tbody>
</table>

* Protein expression was estimated by Western blot and immunofluorescence using CEFs in cultures transfected with the various constructs expressed by the RCAS vector. It is recorded on a scale of 1-4+.

JDV. A c-Jun probe detected the endogenous jun message in CEFs; this message was not detectable in JDV-transformed cells. Unexpectedly, c-Jun message levels were increased in CEFs transfected with the RACS vector alone (data not shown).

A Modified Jun Protein Is Expressed in JDV-transformed CEFs. Three weeks postinfection, an immunoblot was performed with protein extracts from CEFs infected with JDV. Extracts from normal CEFs, from CEFs transfected with JD DNA, and from VC-3-transformed cells served as controls. A polyclonal antiserum directed against the conserved carboxyl-terminal domain of c-Jun was used; this serum detected JunD and Jun with equal efficiency (15) (Fig. 3). JDV-transformed cells expressed two proteins reacting with this antisemur, one of 85 kD and one of 55 kD. The 55 kD protein most likely represents a JunD protein, derived from an intermediate step of the rearrangement. Such intermediate steps were also detected by Southern blot analysis (Fig. 7). Extracts from VC-3-transformed CEFs showed the expected 40 kD v-Jun/c-Jun recombinant protein, and in normal CEFs, a 41 kD band representing the c-Jun protein was detectable.

The c-Jun and c-JunD proteins were not seen and appear to be down-regulated in VC-3- and JDV-transformed CEFs. Similar observations have been made with the corresponding messages (see above). Extracts prepared from JDV-transformed cells 4 weeks after infection showed only the 85 kD polypeptide (data not shown).

Immunofluorescent staining was performed with a monoclonal antibody directed against the avian retrovirus p19 Gag protein. CEFs transformed by JDV gave a nuclear signal (data not shown, but compare Fig. 6, left side). This observation suggests fusion of Gag to JunD, which could result in nuclear translocation of the p19 Gag epitope. Nuclear staining was also obtained with the Jun antibody aPEP2, which efficiently cross-reacts with JunD.

The Genome of JDV Is Rearranged. Southern blots were performed with DNA from JDV-transformed cells, from cells transfected with JD (not transformed), and from control CEFs. The DNA was cut with various enzymes and probed with fragments derived from junD, gag, and the LTR region. A summary of these mapping experiments is presented in Fig. 4. The proviral genome of JDV differed from the original JD construct in several points. The fragments extending from the 3' LTR to the BamHI site at the amino terminus of junD were identical, but restriction enzyme sites farther upstream were miss-

hatched chickens and induced small tumors after a prolonged latent period of 7 weeks. We will refer to this new virus as JDV. As a first step toward understanding its oncogenic potential, we analyzed its genomic structure.

JDV-infected CEFs Contain a New Viral RNA. Fig. 2 shows a Northern blot analysis of CEFs transfected with J D compared to CEFs infected with several stocks of JDV. CEFs transfected with the RCAS vector alone or the transforming v-Jun/c-Jun chimera, VC-3 (16), served as controls. CEFs transfected with RCAS, VC-3, or JD contained the expected three species of retroviral RNA. A 1.7-kb RNA, detectable in all lanes, represented the cellular junD message. This cellular junD RNA appeared to be down-regulated in all jun- and JunD-transformed CEFs. In cells infected with JDV, the 8.8-kb full genome length RNA disappeared, and the two standard subgenomic RNAs of 4.6 and 2.3 kb were missing and replaced by a single 3.5-kb species. This 3.5-kb RNA was also found in JDV-transfected CEFs, albeit in very low quantities. The nature of this 3.5-kb RNA was investigated with retroviral gene-specific probes. It hybridized to gag and LTR fragments but not to a probe of the env region (Fig. 2). It also failed to hybridize to chicken c-Jun, which suggested that jun is not involved in the generation of
ing from the JDV genome. Homologies between JDV and JD were again found in the gag region. The 1.9-kb EcoRI fragment of JD detectable with a junD probe was replaced in JDV with a 3.2-kb fragment (see autoradiograph in Fig. 4). This 3.2-kb fragment also hybridized to a LTR probe and to a probe from the 5' region of gag. Taking into account the RNA and protein data described above, these observations suggest that the two EcoRI sites of the 3.2-kb fragment were located in the LTRs of a new proviral genome.

In order to characterize the sequences between the apparently truncated gag and the junD regions in JDV, the 3.2-kb EcoRI fragment was cloned from a λgt10 library, constructed with size-selected genomic DNA of JDV-infected CEFs. Mapping and sequencing of the isolated clone revealed a new proviral genome in which a 5' fragment of junD is duplicated and inserted between the truncated gag and full length junD sequences (Fig. 5A). The duplication most likely resulted from two recombination events. The first took place between the 5' GC-rich untranslated region of JunD and the equally GC-rich region coding for the polyprolines that are located adjacent to the JunD DNA-binding domain. The two regions show some homology, as seen in Fig. 5B. The second recombination event occurred between GC-rich polyalanine-coding sequences in the duplicated 5' region of junD and a GC-rich sequence in the p10 coding domain of gag (Fig. 5B). The open reading frame of the rearranged junD gene with gag sequences consists of 2064 nucleotides (Fig. 5A). This reading frame could code for the 85 kD protein seen in the immunoblot (Fig. 3). The emergence of the diagnostic 3.2-kb EcoRI fragment could be followed in JD-transfected CEFs during 4 weeks of culture. At the end of this period, JDV was the predominant form of JunD virus.

**A Defective Retrovirus Reconstituted from the EcoRI JDV Fragment Expresses an 85 kD Gag-JunD Fusion Protein and Transforms CEFs.** The 3.2-kb EcoRI fragment cloned from JDV-transformed cells (Fig. 4) contained only partial LTR sequences at the 5' and 3' ends. Complete LTRs were reconstituted by insertion in the RCAS vector using the SacI site upstream of gag and the Clal site upstream of the 3' LTR of RCAS (see “Materials and Methods” for details). The result was a proviral genome that contained complete LTRs but lacked viral coding information and was therefore replication defective. This genome is virtually identical to that of JDV. The construct was transfected into CEFs; controls were cotransfected with JDV and with the RCAS vector which codes for a replication-competent but nontransforming avian retrovirus that could complement the defects of JDV. Two additional controls consisted of CEFs transfected with the replication-competent RCAS vector and the JD construct, respectively. Immunofluorescence of cultures transfected with JDV alone using a Gag antibody showed nuclear staining (Fig. 6, left panel). CEFs transfected with
Independenty Isolated Lines of JunD-transformed Cells Show Similar Genetic Rearrangements of JunD.

JDV is derived from a single JunD-transformed culture. The genetic rearrangement seen in JDV could be unique, or it could be representative of rearrangements that might occur in other JunD-transformed cells. We therefore transfected CEFs with the RCAS constructs JD, JDc8.3, and DDDD (cf. Fig. 1) and then isolated single foci of transformed cells or transformed mass cultures and extracted their DNA for Southern blot analysis. A total of 16 DNA preparations from independent transformants were analyzed; some of the results are presented in Fig. 7. In these tests, the original transfected constructs gave EcoRI/PstI fragments of diagnostic size, 1.6 kb for JD, and 1.1 kb for JDc8.3 and DDDD. Recombination events similar to those described for JDV (Fig. 4) would be expected to increase the size of these fragments. In the case of JD, the expected increase is from 1.6 kb to 2.2 kb in the first recombination, leading to a duplication of sequences in the JunD amino-terminal region. The second recombination step, fusing JunD to Gag, would add another 0.7 kb to generate a 2.9 kb fragment. For JDc8.3, the calculated increases in fragment size are from 1.1 to 1.7 to 2.4 and for DDDD from 1.1 to 1.6 to 2.2.

From JD-transfected cultures, individual foci were picked and grown into mass cultures. DNA analyzed from these transformed CEFs showed rearrangements similar but not identical to those seen with JDV. The variations in the sizes of diagnostic fragments are probably due to differences in the site of crossover within the GC-rich sequences of JunD. Immunofluorescence with a Gag p19 antibody revealed nuclear staining in JD-transformed cells similar to JDV transformants and suggested the generation of Gag-Jun fusion proteins (data not shown). Western blotting with protein extracts detected the expected novel protein bands in the 80 kD region.

In JDc8.3 transfections, we investigated DNA extracted from mass cultures at several time points, from weakly transformed cultures early after transfection to highly transformed cells that predominate when the cells are transferred several times. The early transformants (Fig. 7, Lanes 8 and 9) did not show genetic rearrangements. In intermediate stages (Fig. 7, Lanes 12 and 13), the new 1.7-kb restriction fragment was evident, and in the most highly transformed culture, a further rearrangement step had taken place, resulting in a structure similar to JDV (Fig. 7, Lanes 10 and 11). A similar sequence of events correlating morphological transformation with genetic rearrangement was observed in CEFs transfected with DDDD. Initial and intermediate stages of transformation were characterized by the appearance of multiple new restriction fragments (Fig. 7, Lanes 2 and 3). In the most highly transformed cells, two weak new bands of 1.6 and 2.1 kb were detected in addition to the original 1.1-kb band (Fig. 7, Lane 4). These results suggest that transformation of CEF by JunD is a consequence of genetic rearrangement. The first evidence for new JunD restriction fragments is seen early in culture, sometimes before recognizable morphological transformation has occurred. During two to four passages of the cells, fragments are selected that resemble in size those seen in the original JDV; the larger of these contains both Gag and JunD sequences. The patterns of JunD rearrangements leading

either the replication-competent RCAS or JD construct alone did not show this nuclear localization of Gag but instead displayed cell surface fluorescence, indicating release of infectious virus. Cultures cotransfected with JDV and RCAS showed both nuclear and cell surface fluorescence on the same cells (photograph not included). An immunoblot performed with protein extracts from JDV+RCAS-transformed CEFs revealed a prominent 85 kD protein (data not shown).

Immunofluorescent staining with the Jun antiserum aPEP2 (17) (Fig. 6, right panel) gave nuclear fluorescence in CEFs transfected with JD, JDV, and the JDV+RCAS combination. The signal in JDV-transfected cells was stronger than in JD-transfected cells, possibly because in JDV the aPEP2 epitopes are duplicated. The RCAS-transfected CEFs showed weak nuclear fluorescence, probably representing c-Jun. As described above, RCAS-transfected CEFs also contained elevated amounts of c-Jun message.

CEF s transfected with the replication-defective JDV construct showed low numbers of transformed foci. The efficiency of transformation was greatly increased by cotransfection with the RCAS helper construct, presumably because the release of infectious transforming virus is important in focus formation (Table 1).
to transformation appear to be similar and probably include a duplication and fusion to Gag.

**Duplication of Sequences in the Amino-Terminal Portion of JunD Is Sufficient for the Activation of the Transforming Potential.** The transforming JunD protein of JDV differs from the native, nontransforming version of JunD in two points: (a) It carries a duplication of sequences near the amino terminus. These sequences are homologous to sequences in the A1 transactivator domain of Jun. (b) It is fused to partial Gag sequences. The activation of the transforming potential could then be due to either one of these changes, or it could require both. In order to decide this question, we generated the following constructs (Fig. 1): JDV-N is a deletion mutant of JDV in which the duplicated junD sequences have been removed but the gag sequences have been retained. In JDV-G, the gag sequences are deleted and the portion of the JDV genome representing the duplicated sequences is retained (extending from the 5' BstXI site to the BamHI site in the center of the genome) and joined to 3' sequences derived from wild-type junD. JDV-GA consists of JDV sequences extending from the 5' NaeI site to the central BamHI site. The 3' half of that construct is also derived from wild-type junD. JDV-GA contains only part of the duplicated region of junD characterized by a short glycine-proline rich region; all of gag is deleted. These constructs were tested for transformation of CEFs. They were expressed from the Rc/RSV vector, which does not produce infectious retrovirus and therefore is much more stable genetically than is RCAS. Rc/RSV also carried a G418 resistance marker allowing selection of the transfected cells. CEFs were transfected with the above constructs and with DDDD, JD, and vector alone as controls (Table 2). Cell clones were selected with G418, examined for morphological transformation, and pooled into mass cultures. Immunofluorescent staining showed that junD was expressed in all cells. Only clones transfected with JDV and JDV-G became transformed. JDV-N and JDV-GA did not induce transformation, nor did the controls DDDD and JD. expressed from the Rc/RSV vector. These results suggest that the duplication of junD sequences showing homology to the A1 domain of Jun is sufficient to elicit the transforming potential of JunD. Where the duplicated sequences are completely or partially deleted, as in JDV-N and JDV-GA, the transforming potential is abolished, even if expression is mediated by the RCAS vector (Table 1). Fusion to Gag is not sufficient to convert JunD into a transforming protein.

**JDV Transactivates in CEFs.** The JunD proteins of Fig. 1 were also tested for their ability to transactivate the collagenase promoter containing an AP-1 binding site in the COL-CAT reporter plasmid (18). Rc/RSV constructs expressing the proteins of Table 2 were cotransfected with COL-CAT into CEFs. Protein levels were uniformly high, except for JD and JDV-G, in which the 5' untranslated region of JunD appears to decrease the efficiency...
of translation (13). As described in the preceding paper (15), JunD (DDDD) failed to transactivate, and similar results were obtained with JD and JDV-N (Table 2). Duplication of a glycine- and proline-rich region from the amino-terminal portion of JunD (JDV-GA) led to a marginal increase in transactivation. JDV and JDV-G, however, efficiently transactivated COL-CAT (Table 2). The increased transactivation by JDV was abolished by
the deletions introduced into JDV-N and JDV-GA. These results suggest that the duplication of the conserved amino-terminal JunD sequences in JDV not only is important in transformation but also creates an active transactivation domain.

Discussion
The native chicken JunD protein expressed from the RCAS vector does not induce detectable changes in the growth properties of CEF and, expressed from the Rc/RSV vector, it fails to transactivate the collagenase promoter in these same cells (13, 15). Overexpressed JunD can even function as a transdominant negative in transactivation tests with c-Jun but does not significantly interfere with transformation induced by Jun (15). Although native JunD has little effect in CEFs, a rearrangement of the gene reveals both oncogenic and transactivating po-

Fig. 6. Immunofluorescent staining of CEFs and of CEFs 5 days after transfection with RCAS constructs. A, control CEFs; B, RCAS-transfected CEFs; C, JD-transfected CEFs; D, JDV-transfected CEFs. Proteins were detected with anti-p19 monoclonal Gag antibodies (left) and antibody against a synthetic Jun peptide from the conserved amino terminus (PEP2; Ref. 17) (right).

Fig. 7. Southern blot analysis of genomic DNA, derived from independent DNA transfections or isolates of JunD-transformed cells, probing with a 3’ fragment of junD encompassing the coding region for the leucine zipper and basic domains. The constructs RCAS DDD, RCAS JD, and RCAS JDc8.3 were transfected into CEFs. Single emerging loci were isolated and expanded to cell lines in the case of RCAS JD and RCAS JDc8.3. RCAS DDDDD transected cells were transfected, and DNA was isolated from mass-cultured cells. Black bars beneath the designation of the transfected constructs indicate independent transfection experiments for RCAS DDDDD (Lanes 2-4) or independently picked loci for RCAS JD (Lanes 5 and 14-16) and RCAS JDc8.3 (Lanes 8-13). Genomic DNA from JDV-transformed cells served as reference, isolated at two different time points posttransfection (Lanes 6 and 7). The transformed morphology of the cultured cells was judged from 1+ to 4+ and is indicated at the top of the constructs. The intensities of the 1.6-kb and 2.2-kb fragments in Lanes 4 and the 2.9-kb fragments in Lanes 6 and 7 are faint but clear in the original autoradiograph.
tential. Three clones of JunD, differing in the amount of 5' and 3' nontranslated sequences, have been expressed from the RCAS vector and were carried as infectious avian retroviruses. In the course of a few passages, they underwent genetic rearrangement. In early passages, a multiplicity of different genetic forms can be detected; during further growth, a simpler pattern predominates. The result is the appearance of stable retroviral genomes showing similar but not identical alterations. They code for a JunD variant that is both transforming and transactivating. The remarkable genetic instability seen soon after transfection could be a property of the retroviral vector or of the insert. We believe that both may play a role. Retroviruses show very high rates of genetic recombination (19), and the GC-rich sequences in the insert may increase the probability for nonhomologous crossover. The forces that select for similar genetic rearrangements in independent trans- formators also remain to be identified. They could be related to the cell growth-stimulating properties of the rearranged JunD, but experience with other retroviruses shows that transforming activity does not ipso facto provide a selective advantage for the virus (20).

Deletion and reconstruction analysis suggests that the duplication of sequences in the amino-terminal portion of JunD is sufficient to activate the transforming potential. A segment containing the complete duplication from the 5' region of the JDV genome has been used to replace the unduplicated corresponding region in the junD gene. This construct, JDV-G, then contains only the duplication on an otherwise wild-type junD background. It is transforming and transactivating; therefore, the genetic changes of this segment are necessary and sufficient to elicit the oncogenic and transactivation potentials of junD. Other mutations may occur in the unsequenced 3' portion of JDV, but the activities of the JDV-G construct show that such mutations are not needed for the acquisition of oncogenic and transactivating properties. The deletion constructs JDV-N and JDV-GA lack all or part of the duplicated region, and they are nontransforming and nontransactivating. Gag sequences are not required for the new activities of JDV, but since evidence for Gag fusion was seen in all 16 independently isolated JDV preparations, such fusions may provide a selective advantage.

Table 2  Transformation and transactivation of CEFs expressing JunD from the Rc/RSV vector

<table>
<thead>
<tr>
<th>Transfected construct</th>
<th>Transformationa</th>
<th>Transactivationb</th>
<th>Protein expressionc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rc vector</td>
<td>Ø</td>
<td>1.00 ± 0.06</td>
<td>4+</td>
</tr>
<tr>
<td>Rc VC-3</td>
<td>4+</td>
<td>2.00 ± 0.05</td>
<td>4+</td>
</tr>
<tr>
<td>Rc DDD</td>
<td>Ø</td>
<td>0.77 ± 0.05</td>
<td>4+</td>
</tr>
<tr>
<td>Rc JD</td>
<td>Ø</td>
<td>1.16 ± 0.03</td>
<td>2+</td>
</tr>
<tr>
<td>Rc JDV</td>
<td>3+</td>
<td>14.72 ± 2.31</td>
<td>4+</td>
</tr>
<tr>
<td>Rc JDV-N</td>
<td>Ø</td>
<td>0.82 ± 0.18</td>
<td>4+</td>
</tr>
<tr>
<td>Rc JDV-G</td>
<td>3+</td>
<td>15.44 ± 0.72</td>
<td>2+</td>
</tr>
<tr>
<td>Rc JDV-GA</td>
<td>Ø</td>
<td>1.94 ± 0.11</td>
<td>4+</td>
</tr>
</tbody>
</table>

a Transfected CEFs were selected with G418, and pooled clones were grown into mass cultures and overlayed. Transformation was graded on a scale of 1-4. Ø, not transformed.

b Measured by CAT assay after cotransfection into CEFs with a collagenase promoter-CAT reporter construct.

c Protein expression in cultures transfected with the various constructs in the Rc/RSV vector and estimated by immunofluorescence, 48 h posttransfection.

In the JunD variants and constructs described here, transformation appears to be correlated with transactivation. In contrast, other studies (reviewed in Ref. 15) have emphasized a lack of correlation between these two properties. The conflict between these data is more apparent than real: all investigations on Jun-induced oncogenic transformation agree that the presence of the major transactivation domain is required for oncogenic transformation. Transactivation measured as AP-1 activity is, however, not sufficient for transformation (15, 21–23). The present data are compatible with this interpretation. The specific biochemical activities that allow JunD and Jun to transform cells remain to be identified.

Materials and Methods

RNA Analysis. Isolation, agarose-formaldehyde gel electrophoresis, and Northern blotting of RNA were carried out as described (13). Ten μg of total RNA each were analyzed by blot hybridization. The JunD probe was the carboxyl terminal Clal-HincII fragment (nucleotides 2082–2295) from chicken JunD (13). The retroviral probes derived from Gag and LTR have been described (24). The retroviral envelope probe was a KpnI-Sall fragment, excised from the RCAS vector, subgroup A (25). After hybridization, the filters were washed with 0.1X SSC at 65°C for 1 h.

Proviral DNA Analysis and Molecular Cloning. Preparation of high molecular weight DNA, digestion with restriction enzymes, agarose gel electrophoresis, DNA transfer on nylon sheets, and hybridization to radiolabeled DNA probes have been described recently (13). Washing after hybridization was performed at 60°C in 0.1X SSC for 1 h.

The 3.2-kb EcoRI fragment, representing the proviral genome of the JDV, was cloned from genomic DNA isolated from transformed chicken embryo fibroblasts (JDV 7; see Fig. 1). Sixty μg DNA digested with EcoRI were size fractionated on a 1% agarose gel. Fragments with a size between 2.9 and 3.5 kb were electroeluted, checked by Southern blotting and polymerase chain reaction, and ligated to dephosphorylated arms of Agt10 that had been cut with EcoRI (Stratagene). The ligated mixture was packaged (Gigapack; Stratagene) and 3 × 10⁸ plaques of the unamplified genomic library (complexity, 10⁶ clones) were screened in duplicate with the JunD probe. After hybridization, filters were washed with 0.2X SSC at 65°C for 2 h, and 12 hybridizing clones were observed. The EcoRI inserts of two plaque-purified clones were transferred into Bluescript SKⅠⅠ+ (Stratagene) and analyzed by restriction enzyme digestion. From a 1169-base pair BamHI fragment which encompasses the rearrangements in JDV, segments were further subcloned and sequenced with the Sequenase kit, using single stranded DNA.

Immunoblot Analysis. Protein extracts were prepared from 3 × 10⁸ cells with hot (85°C) 1X SDS gel loading buffer, according to Maniatis et al. (26). After clarification, lysates corresponding to 6 × 10⁷ cells were loaded onto a 10% SDS-polyacrylamide gel as described (27). The separated proteins were electrophoretically transferred onto nitrocellulose (28). Blocking of the filter, antiserum application, and detection were performed with an alkaline phosphatase conjugate kit (Bio-Rad) according to the recommendations of the manufacturer. Jun proteins
were identified with a polyclonal antiserum, directed against the carboxyl-terminal 100 amino acids of c-Jun at a serum dilution of 1:2500.

**Immunofluorescence.** Five days after DNA transfection, cells were passaged onto glass coverslips and incubated overnight. Immunofluorescence of the fixed cells was performed as described (16) with the monoclonal antibodies anti-p19 and PEI2 (17).

**Plasmid Constructs.** A presentation of the plasmid constructs is presented in Fig. 1. The constructs DDDD and VC-3 have been described (15, 16). In order to construct JD, the JunD cDNA clone c7.3 was cut with EcoRI (5’ end) and PstI (nucleotide 2741 in chicken JunD (13)). The fragment was ligated into the corresponding sites of the adaptor plasmid CLA 12 (25). From there, JD was transferred as ClaI fragment into the unique ClaI site of the retroviral RCAS vector, subgroup A (25). In order to clone JD in Rc/RSV (Invitrogen), the EcoRI fragment of c7.3 was first cloned in inverse orientation into the EcoRI site of BlueScript SKI (Stratagene). JD was then released with HindIII-XbaI (restriction sites in the polylinker) and cloned into the HindIII-XbaI sites of the Rc/RSV vector. JDc8.3 is derived from the JunD cDNA clone c8.3 (13). In order to remove the 3’-untranslated sequence, c8.3 was cut with EcoRI (5’-cDNA) and Avall (nucleotide 2361, 9 base pairs downstream from the JunD stop codon) and ligated into the EcoRI and SmaI sites of the adaptor plasmid CLA 12, after blunt ending the Avall site. From there, JDc8.3 was transferred as Clal fragment and cloned into the RCAS vector (subgroup D) cut by Clal. JD and JDc8.3 differ from each other in the nontranslated sequences. JD has 0.20 kb upstream and 0.40 kb downstream; JDc8.3 retains only 0.12 kb upstream and none of the downstream sequences. The JunD viral genome was reconstituted with the 3.2-kb EcoRI fragment from the Agt10 library of JDV-infected CEFs. This fragment was cut with ScaI in the 5’ gag leader sequence and with HindIII at nucleotide 2295 of chicken JunD. A second HindIII-ClaI fragment encompassed the remaining 3’ sequences of junD. In a three-fragment ligation, these segments were joined with the RCAS vector cut by ScaI-ClaI, which then supplied complete LTR regions. From this construct, a SmaI fragment, representing part of the duplicated JunD amino terminus, was deleted to yield JDN. Rc/RSV JDV and Rc/RSV JDV-N were created by ligation of the respective ScaI (5’-gag leader)-ClaI (nucleotide 2082 in chicken JunD) fragments into the Rc/RSV DDDDD vector cut with ScaI (vector polylinker) and Clal (nucleotide 2082 in chicken JunD). JDG was constructed by ligating the BstXI-BamHI fragment containing the duplicated amino-terminal plus a portion of the 5’-untranslated region of the BamHI (nucleotide 1609)-ClaI (nucleotide 2082, chicken junD) fragment of JDN. The ligated fragments were inserted into the vector Rc/RSV JD replacing the BstXI-ClaI internal fragment of that vector. JDG-VA was generated by linking the fragment between the Nael site and the BamHI site in the center of the JDN genome to the BamHI (nucleotide 1609)-EspI (nucleotide 2286) fragment of junD and inserting the fragment in the VVCD plasmid cut by Smal and EspI (15). JDV-VA was released from this construct as XbaI fragment and ligated into the Rc/RSV vector.

**Transfection and Focus Assays.** Polybrene transfection was carried out as described (16, 25). Focus formation was determined 14 days after the transfection. RC/RSV constructs were transfected by the calcium phosphate method, and G418-resistant colonies were generated as described (15). Pooled colonies were cultivated in mass culture. To test for focus formation, 10³ cells were seeded into 35-mm dishes and overlayed after 24 h (100 µg G418/ml). Foci were scored after 5 days.

**CAT Assays.** CAT assays were performed as described (15), using the COI-CAT reporter plasmid (18). The tested constructs were expressed from the Rc/RSV vector (Invitrogen).

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**References**