JunD mutants with spontaneously acquired transforming potential have enhanced transactivating activity in combination with Fra-2

(AAP-1/cellular transformation/spontaneous mutation/direct repeats/transactivation)

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ABSTRACT Although a replication-competent retrovirus that carries junD has no transforming activity in chicken embryo fibroblasts, we have isolated mutant viruses that have spontaneously acquired transforming activity. The molecularly cloned junD genes of three such mutant viruses (T1, T2, and T3) were shown to be responsible for the cellular transformation. DNA sequence analysis indicated that a specific polynucleotide in the junD sequence was tandemly multiplied three times or five times in T1 and T2, respectively. The repeated polynucleotide encodes 16 amino acid residues that are located in a highly conserved region among Jun family proteins. The junD mutation in T3 involved an inversion, a translocation, and nucleotide substitutions that caused drastic amino acid exchanges in another well-conserved region among Jun family proteins. The transcriptional activity of these mutants was analyzed by means of transient expression experiments in F9 cells using a reporter gene containing a single AP-1 binding site. Compared with the wild-type JunD, none of them showed enhanced transactivating activity in the forms of homodimers or heterodimers with c-Fos or Fra-1. However, they did exhibit much higher transactivating activity than the wild type when they formed heterodimers with Fra-2, indicating that the mutated regions function as transactivation domains in a partner-specific manner. Since we have previously reported that there is a basal level of Fra-2 expression in chicken embryo fibroblasts, the results may indicate that protein complexes between JunD mutants and Fra-2 play a crucial role in the cellular transactivation activity.

The c-jun and c-fos protooncogenes were first characterized as the cellular counterparts of the viral oncogenes carried by avian sarcoma virus 17 (1) and Finkel–Biskis–Jinkins murine sarcoma virus (2), respectively. c-jun belongs to a multigene family that includes junB (3) and junD (4, 5), and the jun gene family codes for nuclear proteins that dimerize with the Fos family proteins, such as c-Fos, Fra-1 (6), Fra-2 (7), and FosB (8), to form the transcription factor complex AP-1 (9). Dimerization occurs quite specifically through a leucine zipper structure (10): Jun family members can form low-affinity homodimers and high-affinity heterodimers with the Fos family, whereas Fos-related proteins do not form stable homodimers (11–13). Although these hetero- and homodimers bind to similar DNA binding sites (TGACTCA, AP-1 binding sites), each dimer was shown to have a distinct transcriptional regulatory function, so that transcription can be reversibly and negatively modulated (13–15).

High-level expression of c-jun alone has been reported to cause cellular transformation in chicken primary cultures (16–18) and established cell lines (19). Although junB was shown to have partial transforming activity (20), junD has no transforming activity at all (20, 21). When fos family genes such as c-fos (mouse) (22), fra-2 (chicken) (7), fra-1 (rat), and fosB (mouse) (unpublished results) were introduced into chicken embryo fibroblasts (CEFs) by replication-competent retroviruses, all of them induced cellular transformation of CEFs. With regard to endogenous AP-1 components in logarithmically growing CEFs and quiescent CEFs, the basal level expression of fra-2 (23, 24), c-jun (18, 20, 25), and junD (21) has been reported, whereas c-fos expression is hardly detectable in the absence of growth stimulation.

Although much has been learned about the transforming mechanisms of several oncogenes carried by retroviruses by comparing the structural and biochemical properties of v-onc and c-onc, this approach cannot readily be applied to most of the fos or jun family genes: aberrant expression of most of the members of AP-1 causes clear cellular transformation as described above. In the case of c-jun, for example, initial work was concentrated on the δ region, which is specifically deleted in v-Jun (16) and has been suggested to have several transcriptional functions (26, 27). But its biochemical function in cellular transformation is not so clear because c-jun retains significant transforming activity (17, 18). Several mutational analyses on Jun mutants and the Jun/JunD chimeras have revealed transactivation domains in the N-terminal portion (20) as well as the C-terminal portion (28) of c-Jun, but the most critical domain has not yet been identified. Furthermore, there seems to be no simple correlation between the transforming potential and the AP-1 activity measured in the same cells by means of transient expression experiments (28, 29).

In this report, we first describe the isolation and structural analysis of spontaneous mutants of junD that have acquired transforming activity. Since these gain-of-function mutants altered only a limited region in the coding sequence and have potential advantages for studies of the transforming mechanism by Jun proteins, we next compared the transcriptional regulatory function of these mutants with that of wild-type junD in F9 cells, which are reported to have only a marginal level of endogenous AP-1 activity.

MATERIALS AND METHODS

Cell Growth Conditions and Transfection Assay. CEFs were prepared from 10-day embryos and were maintained in minimal essential medium supplemented with 5% calf serum, 10% tryptose phosphate broth, and 1.0% dimethyl sulfoxide (DMSO) at 38.5°C. The virus infection, virus titer assay, and colony formation in soft agar were essentially the same as described (22). CEFs (1.0 × 10^6 cells per 60-mm plate) were stably transfected with expression plasmids by the Polybrene DMSO method (30), transferred after 2 days, and kept under

Abbreviations: CEF, chicken embryo fibroblast; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase.

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RESULTS

Properties of JunD Virus and Isolation of Mutant Viruses with Acquired Transforming Activity. We have constructed a replication-competent retrovirus that carries the mouse junD gene (JDM1). The virus stock of JDM1 contained a high titer of infectious particle, as judged by interference assay (>10^6 infectious particles), comparable to that of the control virus vector that carries no oncogene (DS3). No significant difference in cellular morphology, growth rate, or saturation density was found between DS3- and JDM1-infected CEFs. These results are in good agreement with the finding of other groups that junD has no transforming activity at all in CEFs (20, 21).

We have noticed, however, that JDM1-infected CEFs formed a slightly larger number of colonies in agar compared with the number of very small background colonies formed by DS3-infected CEFs (Table 1) or uninfected CEFs. Some of the colonies of JDM1-infected CEFs were significantly larger than the background colonies. We also occasionally observed a few foci in monolayer cultures of CEFs fully infected with JDM1 when the cultures were kept under soft agar for >10 days. To test the possibility that the larger colonies formed by JDM1 mutants might have acquired transforming activity spontaneously, 15 colonies were picked up from agar layers, trypsinized, and plated in culture monolayer under soft agar for 3 weeks. Among them, 5 monolayer cultures contained clearly transformed morphology. The culture fluids were recovered and tested for the presence of transforming virus by colony-forming activity assay in soft agar. Two of the virus stocks (nos. 5 and 12) were shown to contain high titers of colony-forming activity, comparable to that of wild-type Rous sarcoma virus (RSV) (SRA) or JH1, which carries the human c-jun gene, whereas the others showed a moderate or low colony-forming activity. These results indicate that the JunD virus can spontaneously acquire transforming activity in the process of viral propagation. For analysis of the transforming mechanisms of Jun proteins, we decided to concentrate on the structural analysis of the virus stocks nos. 5 and 12.

Molecular Cloning and Transforming Activity of the junD Gene Carried by the Mutant Viruses. From the CEF cultures that produced these transforming virus stocks, chromosomal DNA was purified and used as a template for cloning the Bgl II fragment carrying the entire exogenous junD gene by the PCR technique. PCR products were digested with Bgl II, purified by gel electrophoresis, and inserted into the unique Bgl II site of pDS3 in the sense orientation. Transforming activity was tested by transfection and subsequent recovery of the replication-competent virus.

From the proviral DNA that produced the virus stock no. 5, two species of transforming junD clones (T1 and T2) that have slightly different sizes (about 1.2 kb and 1.3 kb) on agarose gel were isolated, whereas one species of junD clone (T3) with transforming activity was isolated from the proviral DNA that produced the virus stock no. 12. The T1 and T2 viruses are very similar to each other and also to the original virus in stock no. 5 in their transforming phenotype. CEFs infected with all of these viruses assumed an elongated spindle shape and formed colonies in agar at a high efficiency. T3 virus and the original virus in stock no. 12 are also similar to each other and CEFs infected with these viruses assumed a rounder phenotype compared with CEFs infected with T1 or T2 virus. It is noteworthy that T1, T2, and T3 have stronger transforming activity than does c-Jun (human), judging from the focal morphology and the size of colonies formed in soft agar.

To confirm further the transforming activity by stable transfection experiments, these junD sequences were inserted into an expression plasmid driven by the RSV long terminal repeat promoter and introduced into CEFs. After 2

<table>
<thead>
<tr>
<th>Virus</th>
<th>Focus number,*</th>
<th>Colony formation per 3 × 10^3 cells</th>
</tr>
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<tbody>
<tr>
<td>DS3 (vector alone)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>JH1 (c-jun)</td>
<td>1.1</td>
<td>86</td>
</tr>
<tr>
<td>JDM1 (junD)</td>
<td>ND†</td>
<td>ND‡</td>
</tr>
<tr>
<td>Clone 5</td>
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</tr>
<tr>
<td>T2</td>
<td>2.9</td>
<td>78</td>
</tr>
<tr>
<td>Clone 12</td>
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</tr>
<tr>
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<td>1.4</td>
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<td>ND‡</td>
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<tr>
<td>T3B</td>
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<td>58</td>
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*CEF.s were kept under soft agar for 7 days after infection and the number of foci was counted.
†When CEFs were kept under hard agar for >10 days, foci became detectable at a low frequency (about 10^3 ffu/ml).
‡Very small background colonies were detectable (5–20) when 10^3 infected cells were plated.
weeks, 6, 11, 15, and 6 foci were formed in 60-mm plate cultures transfected with expression plasmids for c-Jun, T1, T2, and T3, respectively (average of three independent transfection experiments), but no foci were formed at all in those transfected with JunD expression vectors. Each focus was morphologically similar to those formed by the corresponding viruses. Cells transfected with T1 and T2 expression plasmids have more elongated morphology compared with those transfected with T3. These three junD mutants formed bigger and clearer foci as compared with c-Jun (human).

Sequence Analysis of the Transforming junD Mutants. For the structural analysis of these three junD mutants, the entire Bgl II fragments carrying junD sequences were determined. A specific region of junD sequence (397–444 nucleotides from the initiation of the coding sequence) was tandemly multiplied three times and five times in T1 and T2, respectively (Fig. 1A). The repeat unit of 48 oligonucleotides encodes a JunD peptide that completely corresponds to a highly conserved region [region II designated previously (32)] among Jun family proteins (Fig. 2A). Except for these multiplications, only one point mutation, which causes one amino acid exchange, was detected in the coding region of T2. By constructing a chimeric virus between JDM1 and T2 virus, this point mutation was shown to be not necessary for the transforming activity of T2 (data not shown).

T3 junD gene was generated by multiple genetic changes. A polynucleotide composed of 56 bp (279–334) was inserted in the opposite orientation between 319 and 344, which causes a large substitution of 11 amino acid residues to a totally different peptide composed of 21 amino acid residues (Fig. 1B). This kind of inversion has not been frequently detected in retrovirus genomes, but transient switches of the template by the reading strand seemed to have occurred twice during the process of virus genome replication. In addition to this translocation, three continuous nucleotides, AAA (289–291), were changed to GGG, which causes a single amino acid exchange at Lys-97 to Gly. This amino acid exchange at Lys-97, as well as the substituted peptide (from Val-107 to Ser-127), is located in a JunD region that is highly conserved among Jun family proteins (region I) (Fig. 2B). It seems noteworthy that Ser-100 in this region corresponds to Ser-73 in c-Jun (rat) protein, where phosphorylation occurs when such oncogenes as activated c-Ha-ras or v-src are expressed (33). We have constructed a pair of chimeric viruses between JDM1 and T3 viruses—i.e., T3A and T3B (Fig. 2B). T3B virus has transforming potential that is similar to that of T3, whereas the transforming activity of T3A is very limited (Table 1). This result indicates that the large peptide substitution is responsible for the transforming potential of T3.

Transcriptional Regulatory Function of JunD Mutants in F9 Cells. To compare the transcriptional regulatory function of these three JunD mutants with that of the wild type, we have constructed expression vectors of these mutants driven by the RSV long terminal repeat promoter and introduced them into F9 cells together with the reporter plasmid, pcol-

Fig. 1. DNA sequence analysis of junD mutants T1 (A) and T3 (B). Nucleotides were numbered from the initiation of the coding sequence and only the mutated parts are shown. (A) Dots indicate the oligonucleotides that might be involved in the multiplication of the unit sequence. In T2, the same oligonucleotide was repeated five times. (B) Oligonucleotide changes that cause amino acid substitutions are boxed. The upper and lower arrows indicate the 56 oligonucleotides that have been translocated to the opposite orientation. The black circles indicate a single nucleotide change detected in the translocated oligonucleotide.

Fig. 2. Amino acid sequence changes detected in T1, T2 (A), and T3 (B). The black boxes in the wild-type JunD indicate the conserved region among Jun family proteins (I–V) designated previously. Arrowheads in JunD indicate the position of the leucine residues in the leucine zipper structure. (A) The unit of multiplication is shown by shaded boxes. The arrowhead in T2 indicates a single amino acid exchange (Pro-182 of the wild-type JunD to Leu) owing to a point mutation. (B) Amino acids exchanged in T3 or its chimeras are enclosed in black boxes and compared with the wild-type JunD.
TRECAT, which has an AP-1 binding site from the collagenase gene just upstream of the TATA box from the β-interferon gene. As shown in Fig. 3, transactivation activity by the wild-type JunD expression vector alone was low compared with that of c-Jun vector alone. The CAT activity induced by T1, T2, or T3 vector alone is similar to or less than that of the wild type. When combined with c-Fos expression plasmid, JunD expression plasmid significantly elevated CAT activity. This enhancement by c-Fos, however, was not observed in the case of T1 and T2, whereas T3 showed a small enhancing activity. We next cotransfected an expression plasmid of JunD, T1, T2, or T3 with Fra-1 expression plasmid. Fra-1 moderately enhanced the transactivation by all JunD mutants as well as the wild type. The most drastic difference between JunD and its transforming mutants was observed when they were coexpressed with Fra-2. CAT production induced by Fra-2 plus T1, T2, or T3 was much higher than that by Fra-2 plus wild-type JunD and was higher than the level induced by the c-Jun homodimer. In this CAT analysis, we saw a relatively high background level of activity by reporter gene alone (Fig. 4). This activity is probably derived from endogenous JunD or ATF/CREB family proteins in F9 cells (4).

These results indicate that all three mutants have transactivation activities comparable to or slightly less than that of the wild type as far as the homodimers or the heterodimers with c-Fos or Fra-1 are concerned. Furthermore, these results indicate that the three mutants share similar transcriptional regulatory properties that are clearly distinct from those of the wild type: all mutants have much higher transactivation activity specifically when expressed with Fra-2. To examine this specific stimulatory effect of Fra-2 on the JunD mutants more closely, a constant amount of the reporter plasmid was cotransfected with different doses of Fra-2 expression vectors with or without a constant amount of Jun, T1, T2, or T3 expression vectors (Fig. 4). Whereas addition of Fra-2 caused only a slight stimulatory effect on the transactivation activity of wild-type JunD, a drastic stimulatory effect of Fra-2 was observed in all three mutants with a similar dosage dependency.

**FIG. 3.** Transactivation of pcolTRE reporter plasmid by various combinations of JunD derivatives and Fos family proteins that were transiently expressed in F9 cells. F9 cells were cotransfected with a total of 9 μg of DNA containing pcolTRECAT (3 μg) and expression plasmids of c-Jun or JunD or its mutant (3 μg) with or without the expression plasmids of Fos, Fra-1, or Fra-2 (3 μg). Each CAT activity was normalized to the CAT activity of JunD alone. The results of at least three to six independent transfection experiments are indicated as the average relative CAT activity. The relative CAT activity of the reporter plasmid alone (0.83) is indicated by a line.

**FIG. 4.** Transactivation of pcotTRE reporter plasmid by the combinations of JunD derivatives and Fra-2 protein transiently expressed in F9 cells. F9 cells were cotransfected with a total of 9 μg of DNA containing pcolTRECAT (3 μg), the expression plasmids of JunD(c), T1 (e), T2 (a), or T3 (c), and various amounts of expression plasmids of Fra-2. Each CAT activity was normalized to the CAT activity with JunD alone, and results of two independent transfection experiments are indicated as the average relative CAT activity.

**DISCUSSION**

Although aberrant expression of JunD induced no phenotypes of cellular transformation, we have shown here that some changes in a specific part of the JunD coding region are sufficient for the acquisition of the transforming activity. Three of these gain-of-function mutants were analyzed and they were shown to share a transcriptional property that is distinct from that of the wild-type JunD: these mutant JunD exhibit a greatly enhanced transactivating activity specifically in the presence of Fra-2 (Figs. 3 and 4). We have previously reported that Fra-2 forms a stable complex with all of the Jun family proteins in vitro (13) and, further, that Fra-2 is the major component of Fos family proteins in logarithmically growing or quiescent CEFs when analyzed by using an anti-Fos peptide antiserum (7) that is cross-reactive to all murine Fos family proteins. (The presence of the fosB or fra-l genes in the chicken genome has not been proved as yet.) We have also confirmed that levels of fra-2 mRNA in T1-, T2-, or T3-infected CEFs are similar to those in D53-infected CEFs when analyzed by the RNase protection assay (unpublished results). These observations are consistent with the notion that the heterodimer between the mutant JunD and endogenous Fra-2 plays a crucial role in the cellular transformation by these mutants. Our observations also suggest that the mutated regions apparently function as transactivating domains in a partner-specific manner. In the case of Jun protein, however, we have previously reported that coexpression of Fra-2 caused suppression of the transactivation by Jun alone (13). Hence, we think that the transforming mechanisms of c-Jun and the JunD mutants described here are not necessarily the same.

It was recently reported that a replication-competent retrovirus carrying the chicken junD gene acquired a moderate transforming potential by a large rearrangement of the viral genome, generating a replication-defective virus (34). In this mutant, a 0.4-kb S′ fragment of junD is duplicated and inserted between the truncated gag and full-length junD.
sequence. It is of interest that this long duplicated sequence, which was reported to be responsible for the transforming activity, contains the conserved regions I and II, which were drastically changed (T3) or multiplied (T1 and T2), respectively. CEFs infected with this mutant were shown to have elevated AP-1 activity by transient expression experiments. The same group, however, has reported a lack of correlation between transformation and transactivation by jun deletion mutants (29) or jun/junD chimeric constructs (28). This discrepancy might be partially explained by the involvement of different transforming mechanisms between c-Jun and c-Jun/JunD chimeras.

Direct repeat of an oligonucleotide is often observed in the genomes of retroviruses such as the enhancer region located in the long terminal repeat (35). Tandem duplication of a short oligonucleotide of 9 bases is also involved in the generation of v-ros oncogene from its cellular counterpart (36). It should be pointed out that the repeated sequence detected in T1 or T2 starts from GAGCA, which is similar to the 3′ flanking sequence of the unit in the wild-type junD (AAGCA) (Fig. 1A). This homologous sequence might support jumping of the transcription point in the process of viral genome replication for the generation of these multiple repetitions. Since both T1 and T2 sequences were detected in the same transformed cell culture, it might be suggested that these viruses were generated in a stepwise manner and/or that they might be interchangeable at a certain frequency. Such an assumption would imply a relative instability of the genome of these mutant viruses. In fact, however, the transforming activity of T1, T2, and T3 was rigidly confirmed by stable transfection experiments that involved no virus propagation.

Although the potential importance of the conserved regions I and II is revealed by these mutants, the biochemical function in Jun proteins remains to be resolved. It is noteworthy that these regions were reported to contribute to the transactivating activity of Jun (37). It has also been reported that conserved regions 1 and 2 of c-Jun contain HOB1 and HOB2, which have recently been reported to have a low sequence homology to c-Fos and also to function as transactivation domain when both of them were fused to Gal4 protein (38).

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