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Molecular basis of the activation of the tumorigenic potential of Gag–insulin receptor chimeras

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ABSTRACT **A previous study showed that the human insulin receptor (IR) could be activated by insertion of a 3′ portion of the cDNA encoding the β subunit into a retrovirus genome to form a Gag–IR fusion protein. While capable of transforming cells in culture, this IR cDNA-containing virus, called UIR, was not able to induce tumors in animals. Subsequently, we isolated a spontaneous sarcomagenic variant called UIR19t from the parental UIR. UIR19t was molecularly cloned, sequenced, and found to harbor two mutations. A 44-amino acid deletion immediately upstream from the transmembrane domain of the Gag-IR fusion protein removes all the extracellular sequence of the IR remaining in the original UIR construct. In addition, a single nucleotide deletion at the 3′ end results in truncation and replacement of the carboxyl-terminal 12 amino acids by 4 new amino acids. The specific kinase activity of UIR19t is 4- to 5-fold higher than that of the parental UIR. However, no new cellular substrates were detected in UIR19t-transformed cells as compared to UIR cells. Viruses containing either the 5′ or the 3′ deletion mutation were constructed and assessed for their biological function. Our data indicate that the 5′ deletion alone is sufficient to confer tumorigenic ability. We conclude that sequence immediately upstream from the transmembrane domain imposes a negative effect on the transforming and tumorigenic potential of the Gag-IR fusion protein.**

Receptor and cytoplasmic protein-tyrosine kinases (PTKs) constitute the largest family of the known viral and non-virus-derived transforming genes (1, 2). Several of them have been implicated in human malignancies (3, 4). The sequences of the human insulin receptor (hIR) cDNAs (5, 6) indicate that hIR is a typical receptor PTK sharing close homology with the insulin-like growth factor I receptor (7), the *ros* oncogene product of avian sarcoma virus (ASV) UR2 (8), and the sevenless protein of *Drosophila* (9, 10). The mature hIRs exist as dimers consisting of two extracellular α and two transmembrane β subunits (11). Binding of insulin to its receptor leads to substantial activation of PTK activity and metabolic functions, including glucose uptake, lipogenesis and mitosis (11). Despite close homology in the PTK regions of hIR and Ros replacement of the cytoplasmic domain of hIR by Ros did not reproduce the normal physiological responses of the IR, even though the PTK activity of the chimeric receptor could be activated upon binding of insulin (12). Truncation of the extracellular ligand-binding region of the IR resulted in constitutive PTK activity and certain receptor-mediated metabolic functions (13).

To investigate the transforming potential of hIR, we previously constructed a retrovirus by fusing the 3′ portion of the sequence encoding the hIR β subunit to the 5′ *gag* sequence of UR2 (14). This hIR cDNA-containing virus, called UIR, codes for a Gag–IR fusion protein of 75 kDa; it is able to transform chicken embryo fibroblasts (CEFs) but does not induce tumors in chickens (14). Subsequently, a spontaneous variant was isolated by passaging of UIR in CEF culture. This variant exhibits a markedly increased transforming potency and is tumorigenic. We report here the molecular basis of the tumorigenic activation of UIR.

METHODS

Cells and Viruses. The preparation and maintenance of CEFs and colony formation of virus-infected cells followed the published procedure (15). ASV UR2, helper virus UR2AV, and hIR virus UIR have been described (8, 14). UIR19t was derived from UIR19-infected CEF culture and UIR19tr was isolated from UIR19t-induced sarcomas.

Molecular Cloning, Sequencing, and Recombinant Construction. UIR19t and UIR19tr were cloned from circular viral DNAs isolated from infected CEFs, using the λZAP vector (Stratagene) digested at the *Sac* I site as described (16). Once the recombinant phage clone containing the virus insert was obtained, the viral genome was rescued into the phagemid vector pBluescript using XL1-Blue cells and R408 helper phage according to the procedure provided by the supplier of the reagents (Stratagene). The coding regions of UIR19t and UIR19tr were sequenced by the dideoxy method (17) after subcloning the appropriate restriction fragments derived from those regions into M13 phage vectors. UIR19-5d, containing only the 5′ mutation, was constructed by replacing the 3′ *Bst*XI–*Bgl* II DNA fragment [1.3 kilobases (kb)] of UIR19t with the corresponding region from UIR19. In UIR19-3d, the 1.3-kb fragment of UIR19 mentioned above was replaced with the corresponding fragment of UIR19t, thus introducing the 3′ mutation alone into the UIR19 genome.

DNA Transfection and RNA analysis. DNA transfection and Northern analysis of viral RNAs followed published methods (8, 18).

Protein Analysis. Metabolic labeling of virus-infected cells, immunoprecipitation, *in vitro* kinase assay, and Western immunoblot analysis of cell lysates have been described (19, 20). Antibodies against hIR used were monoclonals 15B5, 25D4, and IG2 (21), a polyclonal rabbit serum against the cytoplasmic domain, and rabbit sera, P4 and P5, recognizing a kinase-region peptide and a carboxyl peptide, respectively (22). The anti-phosphotyrosine antibody will be described elsewhere (S. M. Jong and L.-H.W.)

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Abbreviations: ASV, avian sarcoma virus; CEF, chicken embryo fibroblast; IR, insulin receptor; hIR, human IR; PTK, protein-tyrosine kinase.

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RESULTS

Isolation and Biological Properties of UIR19t. UIR transforms CEFs and promotes colony formation of infected cells with delayed kinetics in comparison with bona fide sarcoma viruses such as UR2 or Rous sarcoma virus (14). During propagation of UIR-infected CEFs, however, we noticed on one occasion that the cells exhibited more-pronounced transformed characteristics than the parental UIR. This observation signaled the emergence of a variant(s) with increased transforming potency. The variant was enriched by selecting for the rapidly growing transformed cells in soft agar medium; eventually it was purified by colony isolation. The variant, UIR19t, induces rapid transformation of CEFs (Fig. 1), and efficiently promotes sarcoma formation when injected into newborn chicks (Table 1). Fig. 2 shows representative histological sections of the UIR19t-induced tumors from wing web and pectoral muscle. Both are typical fibrosarcomas consisting of fusiform or stellate tumor cells. The elongated or oval-shaped nuclei of the tumor cells often had multiple prominent nucleoli, and mitotic figures were frequently observed. Tumor cells could be seen infiltrating into and disrupting skeletal muscle fibers, some of which had already degenerated (Fig. 2*B*). The virus isolated from UIR19t-induced sarcomas, called UIR19tr, behaves similarly to UIR19t in promoting morphological transformation and colony formation of infected CEFs (Fig. 1), and in inducing tumors. The transforming potency of UIR19t and UIR19tr with respect to latency of CEF transformation and with respect to incidence and latency of tumor formation *in vivo* are similar to the well-studied spontaneously isolated ASVs such as Rous sarcoma virus and UR2.

RNAs and Proteins of UIR19t and UIR19tr. To investigate the basis for tumorigenic conversion of UIR19, we analyzed the RNAs and encoded proteins of UIR19t and UIR19tr. By Northern analysis, the genomic RNAs of UIR19t and UIR19tr were found to be 100–200 nucleotides smaller than that of the parental UIR19 (data not shown). Confirming that observation, the Gag–IR proteins encoded by UIR19t and UIR19tr were slightly smaller than the parental UIR protein (Fig. 3 *A–C*), whereas the UIR19t and UIR19tr Gag–IR proteins were virtually indistinguishable (Fig. 3*D*). The

Table 1. Pathogenicity of UIR viruses

Virus	Tumor		Cardio- and hepatopathy	
	Incidence	Latency	Incidence	Latency
UIR18/-19	0	—	25%	2–2.5 mo.
UIR19t	90%	2–3 wk	—	—

Approximately 10^5 to 5×10^5 focus-forming units of virus in 0.1 ml of medium was injected into each wing web of 1- to 3-day-old chicks. UIR18 is a parallel clone of the parental UIR19 from which UIR19t was derived. The data for UIR18 and -19 represent pooled results of four injection experiments using five to eight chicks per injection of either virus. Data for UIR19t represent pooled results of three injection experiments. Tumors induced by UIR19t were typical fibrosarcomas. Cardio/hepatopathy invariably resulted in ascitis in 2 to 2.5 months, which was the overt symptom of the illness to be noticed. The affected heart and liver displayed thickening pericardial membrane and liver capsule with multiple focal outgrowth of spindle cells, which nevertheless were not neoplastic and were noninvading. Focal areas of lymphocyte and macrophage infiltration were also noticed on the suprahepatic and pericardiac membranes. In each injection experiment, no chicks from the uninjected control group developed either tumors or hepatocardiac disease.

UIR19tr (Fig. 3 *B* and *C*) and UIR19t (see Fig. 5) proteins appeared to have higher PTK activity when compared to the UIR19 protein by *in vitro* kinase assay. This point will be further described below. The two polypeptides of about 68 kDa and 50 kDa detected in the kinase reaction products (Fig. 3*C*) have not been fully characterized. Although their presence was reproducible, their relative abundance varied among different experiments and appeared to depend on the condition of cell cultures. They could be recognized by anti-IR and anti-phosphotyrosine antibodies (data not shown); most likely they represent the degraded products of the Gag–IR protein. Both RNA and protein data suggested that a deletion is present in the UIR19t and UIR19tr genomes.

Cloning and Sequencing of UIR19t and UIR19tr. The viral DNA genomes of the tumorigenic UIR variants were molecularly cloned from infected cells and the Gag–IR coding regions of both viruses were sequenced. The data revealed that UIR19t and UIR19tr are identical; both harbor a 5′ deletion of 144 base pairs (bp) and a 3′ deletion of 1 bp

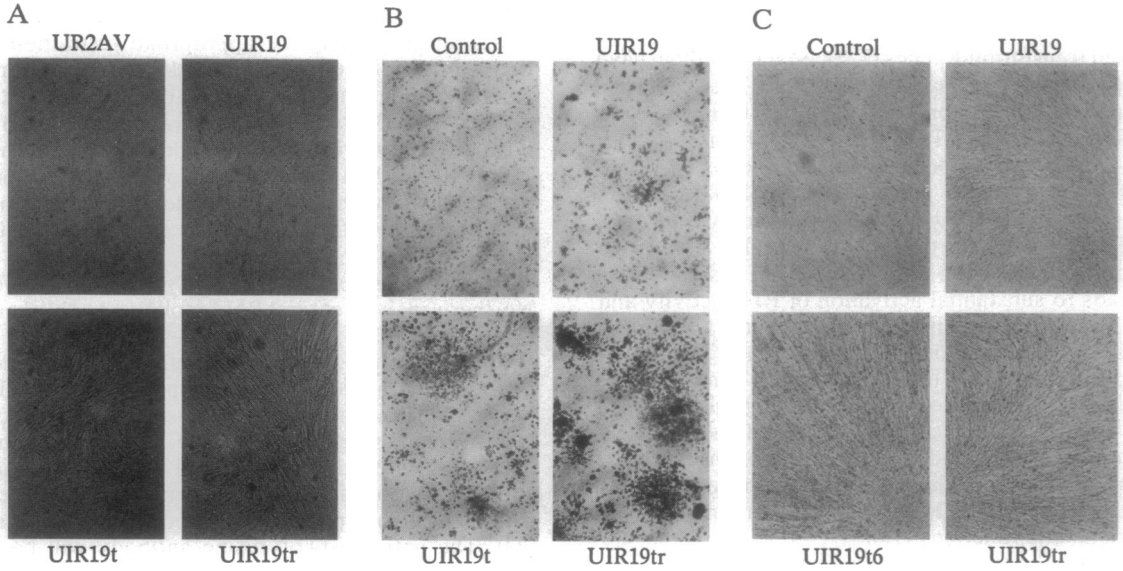

FIG. 1. Transformation of CEFs by UIR19 and its tumorigenic variants. (*A*) CEFs infected with UR2AV or equivalent virus titers of UIR19, UIR19t, or UIR19tr. The cultures represent 10 days after infection. (*B*) Approximately 0.5×10^6 uninfected CEFs or CEFs infected with the virus indicated were plated in soft agar medium. The cultures represent 12 days after cell seeding. (*C*) CEFs were transfected with 1 μg of the indicated molecularly cloned viral DNA together with 0.5 μg of the UR2AV DNA. The cultures represent 2 weeks (two passages) after DNA transfection. UIR19t6 is a molecular clone of UIR19t. (×40.)

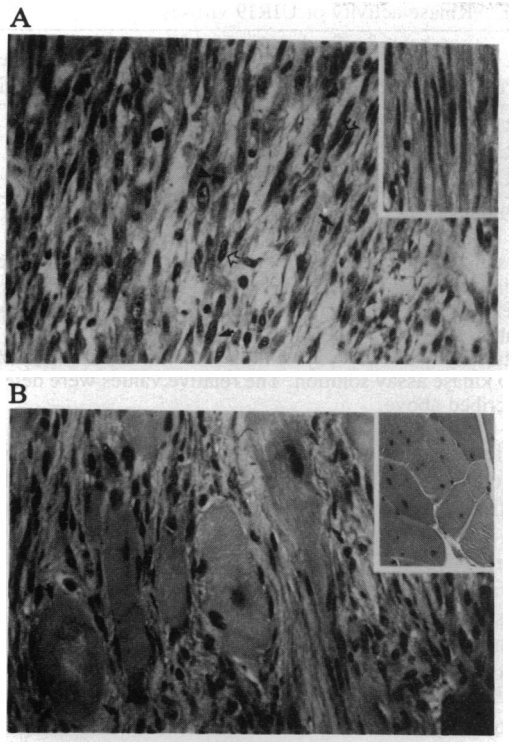

FIG. 2. Histological section of UIR19t-induced tumors. Normal and tumor tissues were fixed and cut into 6-μm sections and stained with hematoxylin and eosin for histological observation. (*A*) Fibrosarcoma taken from wing web. Open arrows indicate cells with prominent multiple nucleoli. Black arrows point to cells with pleiomorphism. Arrowhead shows a nuclear mitotic figure. *Inset* shows normal fibroblasts taken from the capsule of an adrenal gland of the same chicken. (*B*) Fibrosarcoma taken from breast muscle. Infiltration of tumor cells in the muscle fibers and their degeneration are visible. *Inset* shows normal skeletal muscle from the same chicken. (×200.)

(Fig. 4). The 5′ deletion removed all the extracellular IR sequence plus one amino acid of the transmembrane domain in UIR19. This resulted in the joining of p19 and 5 linker

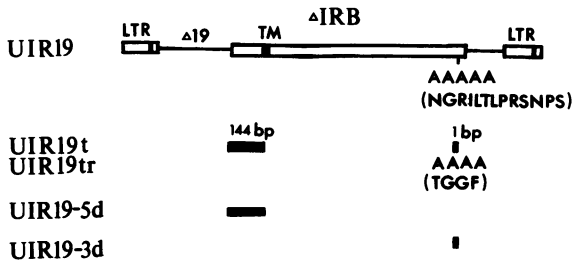

FIG. 4. Structure of the genomes of tumorigenic UIR viruses. Top line represents the structure of the parental UIR19 (14). The hIR β-subunit (IRB) sequence is represented by the open box in the middle of the genome. Cloning and sequencing of UIR19t and UIR19tr and construction of UIR19-5d and UIR19-3d are described in *Methods*. Black bars represent deletions of 144 bp or 1 bp. The new junction sequence in UIR19t and UIR19tr is p19-TSPD-(GDPST)-IIGP-IR. The amino acids in parentheses were derived from the linker region of the plasmid. TM, transmembrane domain; LTR, long terminal repeat.

amino acids directly to the transmembrane domain (Fig. 4). The 3′ single-nucleotide deletion produced a frameshift resulting in truncation of the carboxyl terminus involving replacement of the terminal 12 amino acids by 4 unrelated amino acids (Fig. 4). Molecularly cloned UIR19t and UIR19tr transform cells and induce tumors similarly to their biologically purified parental viruses (Fig. 1 and data not shown).

Protein Analysis of Molecularly Cloned UIR19t and UIR19tr. The properties of UIR19t and UIR19tr proteins were reinvestigated with the molecularly cloned viruses. Again, the size of UIR19t and UIR19tr proteins is slightly smaller than that of UIR19 (Fig. 5 *A* and *B*). As expected, the variant proteins, like that of UIR19, were found to be membrane-associated. However, unlike UIR19, they were not glycosylated, due to the 5′ deletion (data not shown). Furthermore, the carboxyl-end mutation was confirmed by using a peptide antibody, P5, recognizing a carboxyl peptide of hIR (22). The UIR19tr protein was not recognized by this antibody (Fig. 5*B*). The PTK activities of the variant and parental proteins were compared by *in vitro* autophosphorylation and phosphorylation of an arbitrary exogenous substrate, a peptide from the carboxyl-terminal region of lyso-

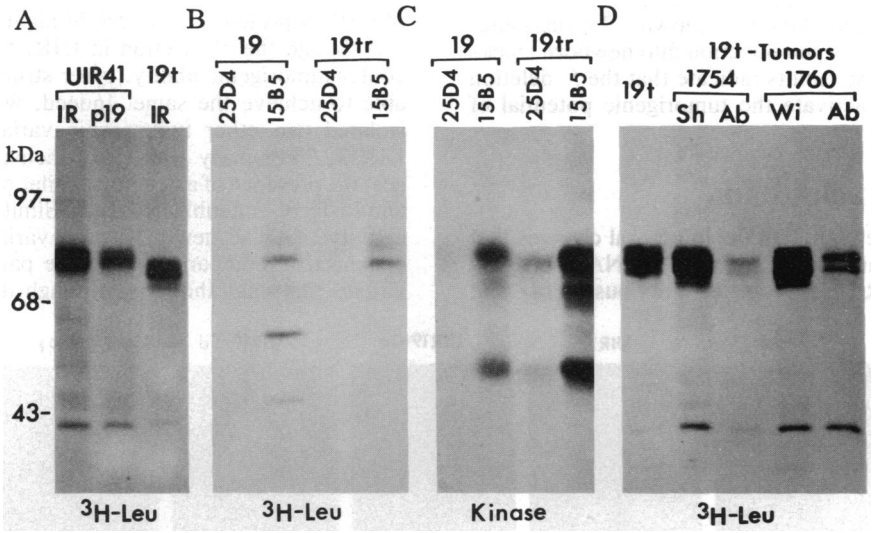

FIG. 3. IR proteins encoded by UIR19 and its tumorigenic variants. CEFs infected with the indicated viruses were extracted directly or labeled with [^{3}H]leucine and then extracted for *in vitro* kinase assay or direct gel electrophoresis after antibody precipitation. UIR41 is a parallel clone of UIR19. Viruses derived from UIR19t tumors taken from two chickens (1754 and 1760) were compared with UIR19t. Sh, Ab, and Wi stand for shoulder, abdominal, and wing web tumor-derived viruses, respectively. The anti-IR antibody 25D4 was used for all lanes in *D*. The particular batch of monoclonal anti-IR antibody 25D4 used for *B* and *C* was not very effective in comparison with 15B5. The antibodies to IR and p19 in *A* and *B* represent anti-IR P4 and anti-p19 2A7, respectively.

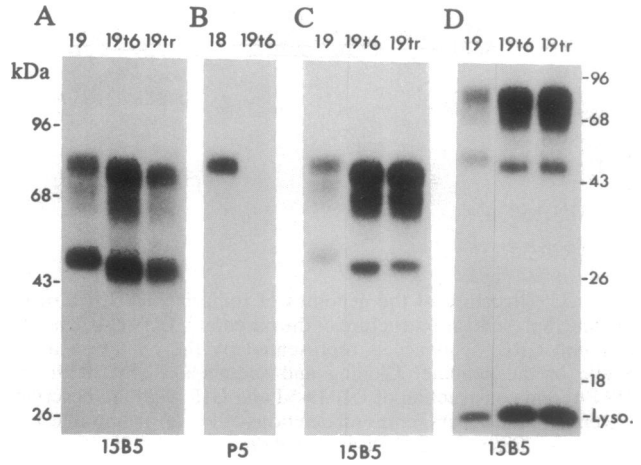

FIG. 5. Analysis of proteins encoded by molecularly cloned UIR19t and UIR19tr. Extracts were prepared from equivalent numbers of uniformly transformed cells, and aliquots were immunoprecipitated with the indicated antibody and subjected to kinase reaction in the absence (*A–C*) or presence (*D*) of lysozyme (Lyso.). Samples in *A–C* were run in SDS/10% polyacrylamide gels, whereas those in *D* were run in a 12% gel.

zyme (Fig. 5 *C* and *D*). Table 2 summarizes the results of Fig. 5 and other data, not shown. The PTK activity of UIR19t and UIR19tr proteins is 4- to 5-fold higher than that of UIR19. However, analysis of phosphotyrosine proteins in infected cells revealed no significant qualitative differences among parental UIR and tumorigenic variants, although cells infected with the tumorigenic variant usually yielded a higher intensity of phosphotyrosine-containing protein bands (data not shown).

Construction and Biological Activity of UIR19-5d and UIR19-3d. To discern whether the 5′ or the 3′ mutation in UIR19t alone or a combination of both was responsible for the tumorigenic conversion, UIR viruses containing either of the two mutations were constructed (see *Methods*). The mutation in each construct was confirmed by nucleotide sequencing. Results of repeated transfection with those viral DNAs showed that UIR19-5d is as potent as UIR19t in transforming CEFs (Fig. 6). By contrast, the transforming ability of UIR19-3d is only slightly increased in comparison with UIR19 (Fig. 6 and data not shown). Furthermore, UIR19-5d is tumorigenic upon injection into newborn chicks (data not shown). These results indicate that the 5′ deletion alone is sufficient to activate the tumorigenic potential of UIR19.

DISCUSSION

In this study, we have identified the structural changes that activate the tumorigenic potential of a hIR cDNA-containing retrovirus called UIR. We proposed previously (14) that

Table 2. Kinase activity of UIR19 viruses

Virus*	Autophosphorylation† Exp. 1	Autophosphorylation† Exp. 2	Lysozyme phosphorylation‡
UIR19	1.0	1.0	1.0
UIR19t6	5.3	4.9	4.0
UIR19tr	6.1	5.4	4.6

*UIR19t6 and UIR19tr here represent viruses derived from molecularly cloned UIR19t and UIR19tr, respectively.
†Equivalent amounts of protein extracts from fully transformed cells were immunoprecipitated and subjected to *in vitro* kinase assay. The relative values were determined by quantitating the signals of the autoradiograms by densitometer tracing.
‡A bacterially derived lysozyme fragment was added to 10 μM in the *in vitro* kinase assay solution. The relative values were determined as described above.

activation of the transforming potential of hIR in UIR virus was due to relief of the ligand-dependent regulation of hIR by truncation of its extracellular domain. In support of this notion was the finding of constitutive hIR activity upon deletion of the bulk of its extracellular domain (13). However, a separate study showed that expression of 5′ truncated hIR cDNAs without joining to any viral sequence was unable to transform CEFs (D.-Y. Du and L.-H.W., unpublished data). Therefore, the *gag* sequence in the transforming protein $p75^{gag-IR}$ of UIR must play some role aside from providing an initiation sequence. Our data indicate that the 5′ deletion in UIR19t is the major change that accounts for the tumorigenic conversion of UIR. Although the 3′ deletion was able to augment the transforming potential slightly, it did not bring about a significant tumorigenic activation (data not shown). The 5′ deletion in UIR19t and UIR19-5d removed IR sequences immediate upstream of the transmembrane domain, resulting in joining of the *gag*-encoded and 5 linker amino acids directly to the transmembrane domain. It appears that sequence immediate upstream of the transmembrane domain imposes a negative effect on the activity of the receptor molecule. This structural constraint could be released by binding of ligand (11) or by deletion of that sequence, as suggested by our current observations, although the two situations may achieve different degrees of activation. Apparently, the Gag–IR of UIR is only partially activated. This notion is supported by the observation that the PTK activity of UIR19t protein is 4- to 5-fold higher than that of UIR19.

Although the 5′ deletion in UIR19t alone is sufficient to confer tumorigenic ability, other structural changes may be able to achieve the same. Indeed, we have independently isolated two other tumorigenic variants from UIR19 and UIR18. Preliminary analysis of their proteins does not suggest the presence of a deletion similar to that in UIR19t. (B.P. and L.-H.W., unpublished data). Similar to UIR19t, the PTK activity of these newly isolated variants is also markedly enhanced in comparison with the parental nontumorigenic viruses (data not shown). Although the enhanced PTK ac-

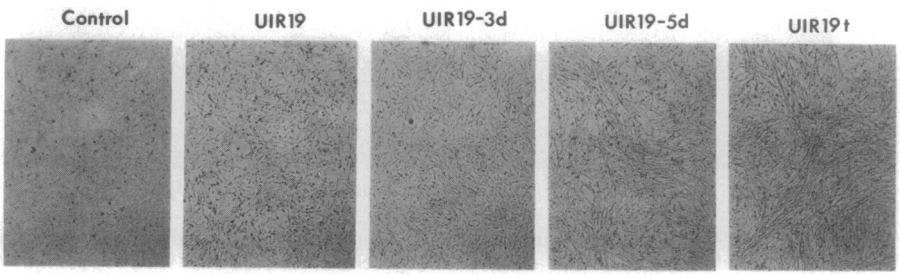

FIG. 6. Transforming potential of UIR19 and its derivatives. The condition for DNA transfection was the same as described in Fig. 1 except that the viral inserts were ligated before transfection to generate nonpermuted viral genomes. This increased the efficiency of virus expression significantly. The cultures represent 11 days (two passages) after DNA transfection. (×40.)

tivity of variant UIR viruses is correlated with their tumorigenic conversion, we cannot exclude involvement of other biochemical properties of these Gag–IR proteins. Nevertheless, we did not detect significant differences in the patterns of cellular substrates found in UIR19- and UIR19t-transformed CEFs.

The $P75^{gag-IR}$ protein, like the $p68^{gag-ros}$ protein (20) is most likely a transmembrane molecule. If so, removal of the extracellular hIR sequence in UIR19t may play a role in evading the host immune response against the transforming protein. This may explain the tumorigenicity of UIR19t and UIR19-5d. However, this would not explain other tumorigenic variants that we have isolated which appear not to contain the large deletion similar to that in UIR19t.

The apparent negative effect of the extracellular IR sequence toward the PTK and transforming activities of the Gag–IR fusion protein could be contributed by glycosylation, or by the sequence per se, or by the combination of them. More defined mutations within that region will be necessary to identify the key structural features. It is possible that these motifs may also play a role in the regulation of signal transduction of native IRs.

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