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Characterization of cDNA Clones for the Human c-*yes* Gene

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Three c-*yes* cDNA clones were obtained from poly(A)$^+$ RNA of human embryo fibroblasts. Sequence analysis of the clones showed that they contained inserts corresponding to nearly full-length human c-*yes* mRNA, which could encode a polypeptide of 543 amino acids with a relative molecular weight (M_r) of 60,801. The predicted amino acid sequence of the protein has no apparent membrane-spanning region or suspected ligand binding domain and closely resembles pp60$^{c\text{-}src}$. Comparison of the sequences of c-*yes* and v-*yes* revealed that the v-*yes* gene contains most of the c-*yes* coding sequence except the region encoding its extreme carboxyl terminus. The region missing from the v-*yes* protein is the part that is highly conserved in cellular gene products of the protein-tyrosine kinase family.

The human cellular *yes* gene, c-*yes*-1, was initially identified as a homolog of v-*yes*, the oncogene of avian sarcoma virus Y73 (47), and was thought to be involved in a case of human gastric cancer (32). The product of the v-*yes* gene, pp90$^{gag\text{-}yes}$, exerts tyrosine-specific protein kinase activity (18). The amino acid sequence of the *yes* specific part of pp90$^{gag\text{-}yes}$ is very similar to that of pp60^{src}, and the *yes* gene is considered to be the most intimate cognate of the *src* gene (19). Although there is accumulating information about the nature of the c-*src* gene product, pp60$^{c\text{-}src}$ (3, 8, 11, 20, 37), not even the structure of the c-*yes* gene product has been elucidated.

At least two v-*onc* genes coding for tyrosine kinases are derived from genes encoding receptor molecules for polypeptide growth factors: the v-*fms* and v-*erbB* genes have been shown to form parts of the genes encoding feline colony-stimulating factor 1 receptor and avian epidermal growth factor receptor, respectively (9, 35). In contrast, the predicted amino acid sequence of c-*src* shows no membrane-spanning region or extracellular domain for ligand binding (39). Nevertheless, pp60$^{c\text{-}src}$ is localized on the inner surface of the plasma membrane and again is thought to be involved in as yet unknown processes of signal transduction at the plasma membrane.

We recently found that c-*fgr*, a cellular homolog of the oncogene of the Gardner-Rasheed strain of feline sarcoma virus (26), and two novel genetic loci named *syn* (33) and *lyn* (45a) in the human genome could encode protein-tyrosine kinases that are very similar to but distinct from the *src* and *yes* gene products. Our primary interest is to determine the functions of these genes that are similar in structure and to find out whether these kinases are involved in the key steps in the process of neoplastic transformation of cells. We have isolated c-*yes* cDNA clones from cultured fibroblasts of human embryo, and here we report the complete structure of these cDNA clones. This information should be useful in determining the normal functions of the c-*yes* gene product.

MATERIALS AND METHODS

Isolation of cDNA clones. A human embryo fibroblast cDNA library was constructed as described elsewhere by using a λgt10 vector system (33). The library (3.7×10^5 plaques) was screened with a 0.9-kilobase-pair (kbp) DNA probe prepared from the human c-*yes*-2 gene, a processed pseudogene of c-*yes*-1 (34). The 1.2-kbp *Bam*HI-*Eco*RI fragment of the c-*yes*-2 pseudogene was freed from repetitive elements by removing a 0.3-kbp *Pst*I fragment containing an *Alu* repeat. Comparison of the nucleotide sequences of the resultant 0.9-kbp pseudogene fragment and genomic c-*yes*-1 DNA confirmed that this DNA fragment reproduced the human c-*yes*-1 coding sequence more faithfully (95% homology) than did the v-*yes* gene (85% homology) (Semba et al., unpublished data). Hybridization was carried out in a solution of 50% formamide, 4× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate), 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-Ethanesulfonic acid (pH 7.0), and 10× Denhardt solution at 42°C. The filters were finally washed with a solution of 0.2× SSC–0.1% sodium dodecyl sulfate at 50°C.

Nucleotide sequence determination. Nucleotide sequencing analyses were performed by the dideoxy chain termination procedure (30). Complementary DNA inserts were recloned into M13 phage vector mp18 and unidirectionally deleted with *Escherichia coli* exonucleases III and VII (46). Some G+C-rich sequences were determined with deoxy-7-deazaguanosine triphosphate in place of dGTP (24).

RESULTS

Isolation of cDNA clones. A cDNA library was constructed with the λgt10 phage vector system and poly(A)$^+$ RNA from cultured human embryo fibroblasts. By use of the human c-*yes*-2 pseudogene probe, we carried out the screening procedures under stringent conditions to eliminate signals caused by other *yes*-related genes (34). By screening about 3.7×10^5 plaques, we obtained three positive clones, designated as λYS-7, λYS-12, and λYS-17. Restriction mapping showed that the clones covered approximately 4.5 kb of the 4.8-kb length of c-*yes*-1 mRNA (Fig. 1).

Analysis of cDNA clones. All four *Eco*RI fragments of the cDNA clones were subjected to unidirectional deletion procedures and subsequent nucleotide sequence analysis. The overlapped portions of each clone showed completely identical nucleotide sequences. The 5′ end of clone λYS-17 was about 50 nucleotides shorter than that of clone λYS-12. Continuity of the sequence across the internal *Eco*RI site was confirmed by comparing the sequence with that of a

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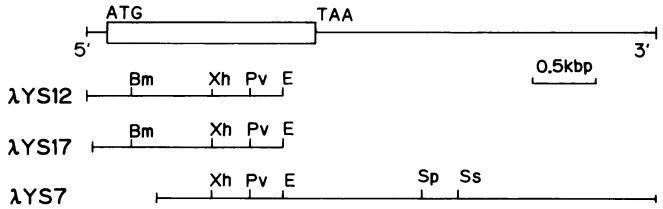

FIG. 1. Restriction maps of cDNA clones. The line shown above is a scheme of total c-*yes* mRNA. The open box indicates the predicted coding frame. The mapped restriction sites are abbreviated as follows: Bm, *Bam*HI; E, *Eco*RI; Pv, *Pvu*II; Sp, *Sph*I; Ss, *Sst*I; Xh, *Xho*I.

genomic clone of human c-*yes*-1. The sequence of 4472 nucleotides covered by clones λYS-12 and λYS-7 contains an open reading frame specifying a 543-amino-acid polypeptide initiated with methionine at position 163 (Fig. 2). Although precise location of the 5′ terminus of the c-*yes* mRNA was not examined, the codon seems to be a bona fide initiating codon for the polypeptide, because there is no additional ATG triplet preceding this one, and the reading frame begins just behind the termination codon at the nucleotide at position 4.

The relative molecular weight of the deduced polypeptide, 60,801, is very similar to those of the c-*src* and *syn* proteins. The amino acid sequence of the polypeptide from residue 91 to the carboxy-terminal residue 543 that includes the suspected kinase domain shows 96% identity to the corresponding region of the v-*yes* protein (excluding the eight carboxy-terminal amino acids), 84% identity to that of the chicken c-*src* protein, and 82% identity to that of the *syn* protein. In addition, the sequence retains a high degree of homology to the v-*yes* protein, even in its amino terminal moiety (residues 1 to 90), except in some parts (residues 34 to 47; see below), whereas the same regions of the c-*src* and *syn* proteins show less homology to the v-*yes* protein (Fig. 3). These data seem to validate the conclusion that the clones represent the c-*yes*-1 genetic locus, the human homolog of the v-*yes* gene.

As in other tyrosine kinases, a well-confirmed consensus sequence, Gly-X-Gly-X-X-Gly (residues 284 through 289), and Lys-305 for ATP binding are conserved in the polypeptide (16, 44). Moreover, Tyr-426 corresponds to Tyr-416 of $pp60^{c\text{-}src}$, which is the target of the autophosphorylation reaction (36). These features suggest that the c-*yes*-1 protein is a protein-tyrosine kinase.

$pp60^{c\text{-}src}$ was reported to be phosphorylated at Ser-17 by A kinase (6) and at Ser-12 by C kinase (13). But the serine or threonine residues at homologous positions in the c-*yes* protein cannot be identified because of the limited degree of homology between $pp60^{c\text{-}src}$ and the c-*yes*-1 protein in their amino-terminal regions.

Myristic acid is attached to Gly-2 of $pp60^{c\text{-}src}$ through an amide bond, whereas the initiating methionine of the protein is removed in the mature form (4, 17). In the predicted c-*yes*-1 protein, the initiating methionine is also followed by glycine. This Gly-2 therefore may be the myristylation signal in the human c-*yes* protein, whereas the corresponding Gly-278 could be silent in the v-*yes* protein. Studies are required on whether the c-*yes* protein is myristylated at the Gly-2 in mature form.

The newly identified sequence of 12 amino acids at the carboxyl terminus of the peptide is very similar to those of the human and chicken c-*src* proteins (1) and identical to that of the human *syn* protein (33). The sequence contains a tyrosine residue (Tyr-537) corresponding to Tyr-527 in $pp60^{c\text{-}src}$, which is suggested to be phosphorylated for negative regulation of its kinase activity (7).

Comparison with the v-*yes* gene. Comparison of the cloned cDNA sequence with that of the v-*yes* gene revealed that the entire coding sequence of the c-*yes*-1 gene is included in the v-*yes* gene except for the sequence encoding the carboxyl terminus of the protein. Nucleotide sequence homology between v-*yes* and human c-*yes*-1 was clearly observed downstream of the second letter in the codon of the initiating methionine (Fig. 2). It appears probable that the chicken c-*yes* gene product is initiated from the same site with human c-*yes*-1, and methionine at the initiation codon seems to be replaced by valine in the v-*yes* gene. In this case, codon GTG for valine could be the result of a transition event from adenine to guanine nucleotide in the viral genome. Three additional stretches of homology, A, B, and C, are observed between the 5′ noncoding region of human c-*yes*-1 cDNA and the corresponding part of the v-*yes* gene (Fig. 2). First, there is a homologous sequence A of eight nucleotides a little upstream of the initiating methionine codon. Interestingly, a sequence similar to the left seven nucleotides is located at the 3′ border of the first exon of the chicken c-*src* gene (39). Since the exon-intron architectures of the c-*src* and c-*yes*-1 genes are identical so far as examined (Semba et al., unpublished data), this seven-nucleotide sequence of c-*yes*-1 cDNA is likely to be the exact 3′ border of the first exon of the human c-*yes*-1 gene. Just upstream from this site, the sequences of v-*yes* and human c-*yes* are both G+C rich, and the homologous region B of the two is a G+C stretch of 10 identical nucleotides in this G+C-rich region. There is a third homologous sequence, C, of 17 nucleotides in the region corresponding to the 5′ recombination site of the chicken c-*yes* and viral sequences, which is discussed below. Although these homologous sequences may suggest that the v-*yes* in this part represents the 5′ noncoding sequence of chicken c-*yes* mRNA, we cannot tell decisively at present whether it includes some intron structures.

To our surprise, in spite of the partial conservation of the nucleotide sequence (55% homology) from position 261 to 302, the deduced amino acid sequence (residues 34 to 47) of human c-*yes*-1 is completely different from the corresponding sequence of v-*yes* (Fig. 2 and 3). To avoid possible mistakes in sequencing, we confirmed the nucleotide sequence of this region by sequencing two independent cDNA clones (λYS-12 and λYS-17) and a v-*yes* cloned DNA.

As mentioned above, nucleotide sequence analysis disclosed an array of 12 amino acids in the carboxyl terminus of the c-*yes*-1 protein that is very similar to those of other tyrosine kinases. Our preliminary data on the chicken c-*yes* cDNA sequence showed that the chicken c-*yes* protein had the same carboxy-terminal 12 amino acids as those of the human c-*yes*-1 protein, except that Glu-541 in human c-*yes*-1 is replaced by aspartic acid in chicken c-*yes* (Sukegawa et al., unpublished data). In the v-*yes* gene, the extreme 3′ terminus of the chicken c-*yes* coding sequence that specifies these 12 amino acids is replaced by the viral sequence for the *env* protein gp37 in a frame that differs from that of the original protein. At the site of this replacement, both the nucleotide sequences of the chicken and human c-*yes* and that of the Prague strain of Rous sarcoma virus (Pr-RSV) share a homologous sequence of 10 nucleotides, which is discussed below. Therefore, we conclude that by this replacement the carboxy-terminal eight amino acids in the c-*yes* protein are replaced by three different amino acids in

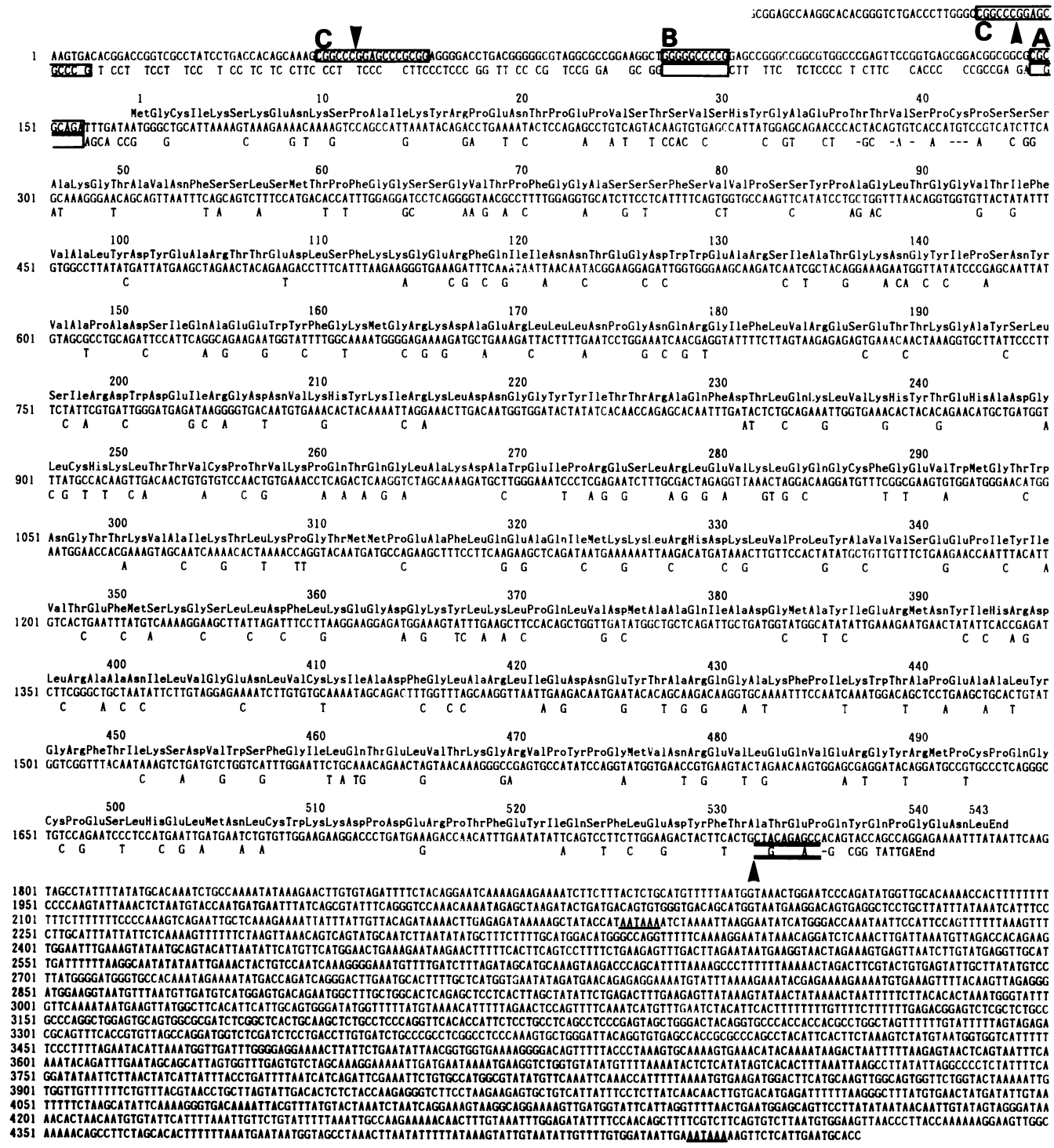

FIG. 2. Complete sequence of the human c-*yes*-1 cDNA and corresponding portion of the v-*yes* gene. The sequences of the human c-*yes*-1 cDNA and the v-*yes* gene are aligned in accordance with the sequence homology between them. Nucleotides of the v-*yes* coding sequence are shown only where they are different from those of the cDNA. The sequence of the v-*yes* are shown a little longer than that of the 5′ end of the cDNA to exhibit the additional sequence homology 'C' between the two (the single line of sequence at the top of the figure). Nucleotides of the cDNA are numbered on the left, and deduced amino acids are also numbered from the initiating methionine. Three homologous sequences between the 5′ noncoding region of human c-*yes*-1 gene and the corresponding part of v-*yes* are boxed and marked with A, B, and C. Arrows on the v-*yes* sequence indicate the postulated 5′ and 3′ recombination sites between chicken c-*yes* and the viral sequence described previously and the site corresponding to the 5′ recombination in the human c-*yes* is also indicated by an arrow at the box C (19). A 10-nucleotide stretch of homology between human c-*yes*-1 and v-*yes* at the 3′ recombination site is underlined (see the text). The sequence AATAAA in the 3′ non-coding region is underlined.

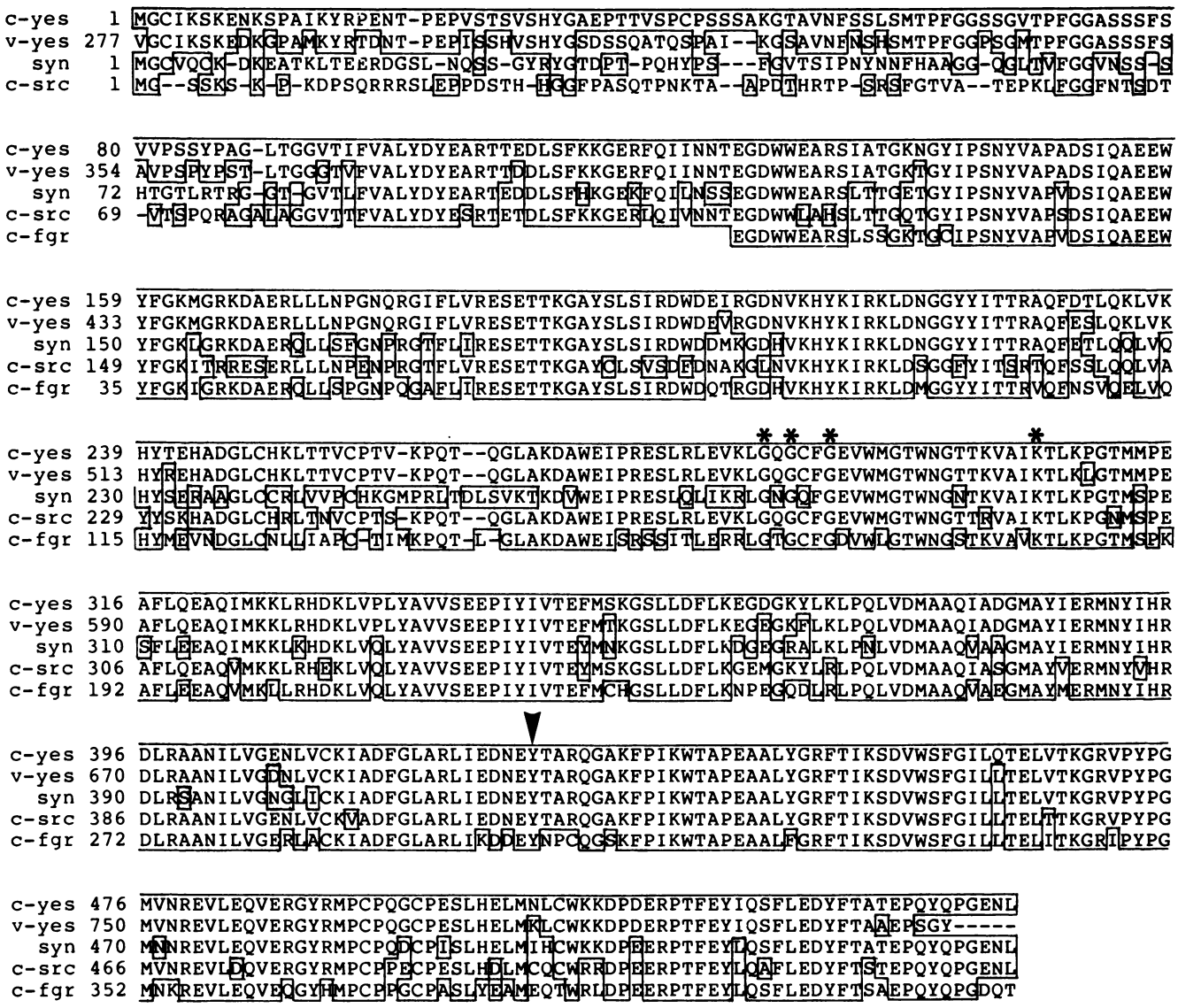

FIG. 3. Comparison of the amino acid sequences of human c-*yes*-1, v-*yes* (19), *syn* (33), chicken c-*src* (39), and human c-*fgr* (26, 28). Amino acids identical to those of human c-*yes*-1 are boxed. Amino acids of each protein are numbered on the left. The amino-terminal sequence of the human c-*fgr* protein is not available. Asterisks indicate the conserved amino acids supposed for nucleotide binding. The arrowhead indicates the target residue for the autophosphorylation reaction.

the v-*yes* protein, and that Thr-533 in the c-*yes* protein is replaced by alanine in the v-*yes* protein (Fig. 3 and 4).

Besides the alterations described above, there are many other amino acid differences between the human c-*yes*-1 protein and the corresponding portions of the chicken-derived v-*yes* protein. Our limited degree of data on chicken c-*yes* cDNA so far available have demonstrated that the amino acid differences in the part downward from His-513 in the v-*yes* protein, other than at Lys-583 and at the C terminus, are due to species differences and therefore irrelevant to the transforming ability of the v-*yes* gene. The Pro-312 in the human c-*yes*-1 protein, which corresponds to Lys-583 of v-*yes*, is conserved in the chicken c-*yes* protein (Sukegawa et al., unpublished data). Complete nucleotide sequencing of chicken c-*yes* is necessary to determine whether the rest of the amino acid differences are important for the transforming ability of v-*yes*.

Sequences at recombination junctions in the v-*yes* gene. Avian sarcoma virus Y73 has incorporated the chicken c-*yes* gene into its genome by at least two successive events of recombination between the c-*yes* gene and viral sequences. The 5′ recombination has taken place in the coding sequence for the *gag* precursor protein, and the 3′ recombination has occurred in the viral *env* sequence. Both recombination sites on the virus genome were suggested in our previous study from a comparison of the v-*yes* sequences with those of Pr-RSV (19, 31). Comparison of the nucleotide sequence of human c-*yes*-1 with that of Pr-RSV revealed that the two genes shared short stretches of nucleotide sequences in regions corresponding to the 5′ and 3′ recombination sites (Fig. 2 and 4). In the region corresponding to the 5′ recombination junction, which seems to have occurred in the 5′ untranslated region of the c-*yes* gene, the human c-*yes*-1 and v-*yes* sequences have a homologous sequence of 17 nucleo-

tides (15 of 17 nucleotides are identical). The *gag* sequence of Pr-RSV also shows homology with the 5′ half of this 17-nucleotide stretch (8 of 9 nucleotides are identical). This stretch of homology extends six nucleotides further upstream from the point at which the 5′ recombination is thought to have occurred. Downstream from the point of the tentative 3′ recombination junction there is a 10-nucleotide stretch which shows partial homology in human c-*yes*-1 and Pr-RSV (8 of 10 nucleotides are identical). The 10-nucleotide sequence of Y73 in this region completely matches that of Pr-RSV, which is a part of the *env* gene specifying gp37. Preliminary sequencing data on chicken c-*yes* cDNA showed that 9 of 10 nucleotides in this region of the chicken sequence are identical with those of Pr-RSV (Fig. 4).

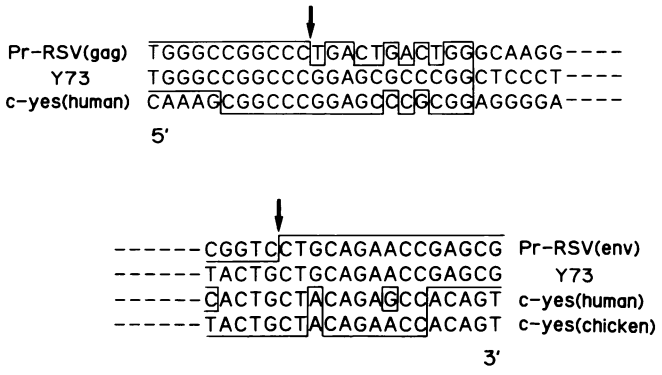

FIG. 4. Comparison of the nucleotide sequences of c-*yes*, Y73, and Pr-RSV around the recombination junctions. Nucleotides identical to those of Y73 are boxed. The two arrows indicate the sites of 5′ and 3′ recombination junctions between chicken c-*yes* and the viral sequence described previously (19).

DISCUSSION

Cellular protein-tyrosine kinases are classified into two groups. One group includes receptor molecules for polypeptide growth factors. To date, five receptor molecules of this type are known: the receptors for insulin (17, 40), somatomedin C (29), epidermal growth factor (5), platelet-derived growth factor (10), and colony-stimulating factor 1 (35). In addition, the *trk* (*oncD*) (23), *erbB2* (2, 45) and c-*ros* (25) genes are suggested to encode receptor-type proteins. In contrast, $pp60^{c\text{-}src}$ and $pp56^{lsk\text{-}tck}$ (22, 43) have no structures characteristic of transmembrane receptor molecules; they have no stretch of hydrophobic amino acids for spanning the membrane and no extracellular domain for ligand binding. Both molecules are acylated by myristic acid and anchored to the inner surface of the plasma membrane (12, 21, 42). Although they are not receptor molecules, these nonreceptor-type kinases, constituting the second group of cellular protein-tyrosine kinases, are believed to be essential as effectors or modulators of some signal transduction processes occurring at the plasma membrane. The predicted products of the *syn* and *lyn* genes are both classified in this group. We have isolated and characterized cDNA clones for the human c-*yes*-1 gene and demonstrated that the product of the c-*yes*-1 gene also belongs to this nonreceptor type of protein-tyrosine kinase family.

The abilities of many viral oncogenes to transform cells have been ascribed to their protein-tyrosine kinase activities. These viral tyrosine kinase genes are activated versions of normal cellular counterparts incorporated into the virus genome. The transduction of the cellular gene into a viral genome inevitably causes structural alterations of the cellular gene product. Subsequent cycles of virus replication may well allow additional mutations in the coding sequence of the incorporated cellular gene. These alterations are considered to account for the acquisition of transforming ability of the viral oncogenes. The nucleotide sequence of the human c-*yes*-1 cDNA revealed major alterations that the chicken c-*yes* gene had undergone during the processes of its incorporation into the viral genome. The carboxy-terminal sequence of eight amino acids of the c-*yes*-1 protein, which includes the tyrosine residue (Tyr-537) that is thought to be involved in negative regulation of kinase activities (7), is replaced by different amino acids in the v-*yes* protein. The 5′ recombination event resulted in joining of the viral *gag* sequence and the suspected 5′ noncoding exon or intron sequence of the chicken c-*yes* gene. Similar recombinations are suggested in the cases of v-*fps* and v-*myc*, in which 5′ untranslated or intron sequences are used to generate the viral oncogenes (14, 27). The extraneous portion of the fused protein might alter the biological specificities of the authentic cellular gene product, such as its substrate specificity and subcellular localization. Besides these changes, there are many differences between the amino acid sequences of the human c-*yes*-1 and v-*yes* proteins. Some of these alterations could be responsible for the transforming ability of the v-*yes* protein, but the exact mode of activation of chicken c-*yes* cannot be defined until the entire structure of chicken c-*yes* is determined.

The mechanism by which the cellular sequence is incorporated into the viral genome is not fully understood, although several models have been suggested (38, 41). Comparison of the nucleotide sequence of human c-*yes*-1 cDNA with that of Pr-RSV revealed remarkable homologies between the two at both of the recombination sites postulated for the v-*yes* gene. Van Beveren et al. reported that the sequence homologies between the incorporated cellular gene and the viral genome were also observed in the case of *fos* in Finkel-Biskis-Jinkins strain of murine sarcoma virus (41). They proposed three possible modes of incorporation of the cellular sequence into the viral genome: (i) double crossover between viral and cellular DNAs, (ii) 5′ recombination at the DNA level and 3′ recombination at the RNA level during reverse transcription, and (iii) both the 5′ and 3′ recombinations at the RNA level during reverse transcription. The second mechanism, which appears to be widely applicable to many other cases of retroviral transduction of the cellular sequence, could be facilitated by the sequence homologies described here, although the homologies cannot exclude either of the other mechanisms. We have confirmed that the chicken c-*yes* sequence retains the homology at the 3′ recombination site. We are now trying to isolate a full-length chicken c-*yes* cDNA clone to examine whether the 5′ sequence homology described here is also conserved in the chicken genome.

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