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Interaction of the Adenovirus L1 52/55-Kilodalton Protein with the IVa2 Gene Product during Infection

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The adenovirus L1 52/55-kDa protein is expressed both in the early and late stages of infection, raising the possibility that it has multiple roles in the viral life cycle. To obtain possible insights into these roles, the yeast two-hybrid system was used to examine the interactions of the 52/55-kDa protein with viral and cellular factors. cDNA expression libraries from human 293 cells at both early and late stages of adenovirus type 5 infection were constructed and screened, with the 52/55-kDa protein being used as bait. Characterization of positive clones revealed that the adenovirus IVa2 gene product interacted specifically with the 52/55-kDa protein. In addition, the IVa2 protein was shown to interact with a bacterial glutathione *S*-transferase–52/55-kDa fusion protein in vitro, further supporting the finding with the yeast two-hybrid system. Finally, coimmunoprecipitation studies confirmed that the 52/55-kDa protein and IVa2 polypeptide interact specifically during the course of adenovirus infection. A potential role for the IVa2–52/55-kDa protein interaction in the regulation of transcription from the major late promoter and in viral assembly is discussed.

Two classes of mRNAs, early and late, are produced during adenovirus infection. The late transcripts are further divided into five families of 3′ coterminal transcripts, L1 to L5 (9). The L1 transcripts encode the IIIa and 52/55-kDa proteins. Analysis of an adenovirus harboring a temperature-sensitive mutation in the 52/55-kDa protein (H5ts369) indicates that the protein is required for assembly of viral particles (15). Unlike the other families of late transcripts, the L1 mRNAs are detectable very early after infection has commenced (1, 18, 20), and the 52/55-kDa protein is detectable 6 or 7 h postinfection (16). The appearance of the 52/55-kDa protein before viral DNA replication has begun or any viral structural proteins have been produced suggests that it may be responsible for some activity early in the infectious process.

We reasoned that involvement of the 52/55-kDa protein in viral assembly or any additional functions of the 52/55-kDa protein during early infection would involve specific protein-protein interactions. To examine these possibilities, the 52/55-kDa open reading frame (ORF; amino acids [aa] 2 to 416) was fused to the LexA DNA binding domain in the yeast expression vector pEG202 (10) and used as bait in a yeast two-hybrid screen. To ensure the detection of early- and late-stage-specific interactions, cDNA expression libraries were prepared from poly(A)$^+$ RNA isolated (6) from human 293 cells at the early (1-β-D-arabinofuranosylcytosine [araC]-treated) (4, 8) or late (18 h postinfection) stage of infection. Libraries were constructed in the yeast vector pJG4-5 (12) with the ZAP-cDNA synthesis kit (Stratagene Cloning Systems, La Jolla, Calif.), and their characteristics are described in Table 1. A total of 3×10^7 CFU from each library was analyzed for interactions as described previously (10). Positive clones were designated by library (E, early; L, late) and the order in which they were isolated.

Yeast colonies (n = 240) from each cDNA library screen were tested for their ability to express proteins that interact with the 52/55-kDa protein. Seventy-nine percent and 94% of the clones from the early and late libraries, respectively, remained positive for interactions with the 52/55-kDa protein. Analysis of 83 early and 183 late cDNA inserts by PCR amplification and digestion with *Hae*III revealed that 48% of the early and 74% of the late clones contained identical or nearly identical cDNA inserts. Further characterization of the cDNA inserts revealed slight differences in the 5′ portions of these clones. One of the shortest (L60) and one of the longest (E3) clones, as well as two intermediate-length clones (E53 and L112), were retransformed into *Saccharomyces cerevisiae* harboring either the 52/55-kDa bait or one of two nonspecific baits: the *Drosophila* bicoid protein (10) or a portion of the simian virus 40 large-T antigen (aa 5 to 172) (15a). All four of the cDNA clones remained positive for interactions only in yeast cells expressing the 52/55-kDa protein.

Sequence analysis of these clones revealed that their 3′ ends terminated at the reported cleavage-polyadenylation site for the adenovirus IVa2-E2B mRNAs and that they extended different distances 5′ from the polyadenylation signal (Fig. 1). The 5′ end of the smallest clone (L60) maps to nucleotide 5357. Analysis of the 5′ junction of L60 indicated that it would produce an IVa2 fusion protein containing the C-terminal 421 aa of the IVa2 gene product. Analysis of the three remaining clones showed that their 5′ ends mapped within 50 nucleotides of each other. All three of these clones were predicted to produce fusions with the IVa2-E2B DNA polymerase ORF. The ORFs of the DNA polymerase and IVa2 genes overlap and are in frame with each other in this region of the genome and diverge when an IVa2-specific splicing event generating the mature IVa2 mRNA occurs. The resulting IVa2 ORF has only 5 aa in common with the DNA polymerase gene (Fig. 1).

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TABLE 1. Characteristics of cDNA libraries constructed for use in the yeast two-hybrid system

Library	No. of primary transformants[a]	% Recombinants[b]	Avg size of insert[c]	No. of yeast primary transformants[d]
Early	3×10^6	83	1.1	1×10^7
Late	2×10^6	94	1.4	4×10^6

[a] Number of colonies obtained upon transformation into XL1B-MRF′ cells.
[b] Percentages of plasmids analyzed that contained cDNA inserts.
[c] Approximate sizes (in kilobases) of inserts observed by agarose gel electrophoresis.
[d] Number of colonies obtained upon transformation of plasmid cDNA libraries into yeast strain EGY48 (7) expressing the LexA–52/55-kDa bait protein.

Because the three clones E3, E53, and L112 all mapped upstream of the IVa2 intron, the splice junction was sequenced to determine if these clones produced a IVa2 or a DNA polymerase fusion protein. In all three cases, the IVa2 intron had been removed, indicating that in the yeast two-hybrid system, the adenovirus 52/55-kDa protein interacts with the viral IVa2 gene product.

Sequence analysis of the IVa2 clones isolated in the two-hybrid screen revealed that several could produce fusion proteins that contained a small number of amino acids specific to the DNA polymerase gene. Several points argue that the presence of these amino acids is coincidental and that they do not contribute to the interaction with the 52/55-kDa protein. First, these clones have had the IVa2 intron removed, suggesting that they arose from IVa2-specific transcripts. Second, the 5′ end of L60 maps downstream of the IVa2 intron and does not contain any DNA polymerase coding ability, indicating that DNA polymerase sequences are not required for the IVa2–52/55-kDa protein interaction. Finally, we did not detect any E2B-DNA polymerase-specific cDNAs in screens of either the early or late libraries. Sequence analysis also revealed that the 5′ end of clone E3 mapped 15 nucleotides upstream of the published 5′ cap site of the IVa2 mRNA (nucleotides 5841 to 5836).

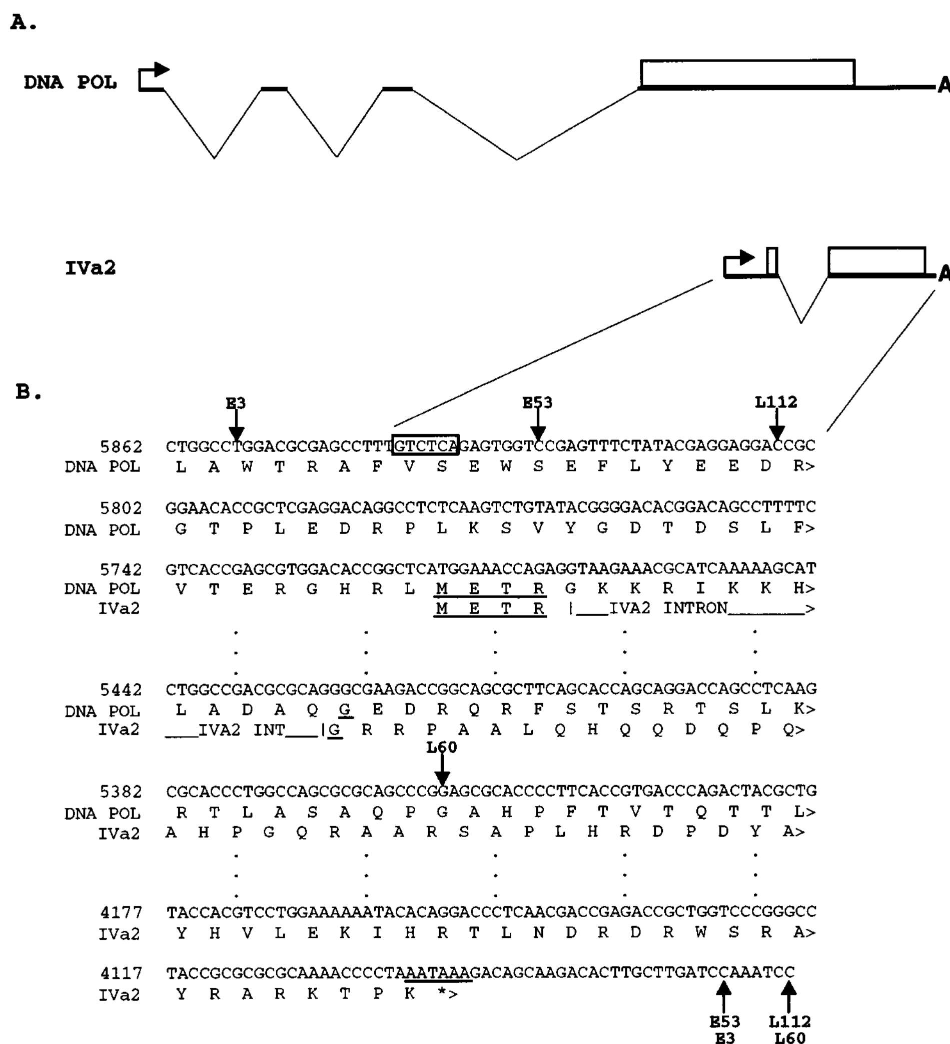

FIG. 1. Sequence analysis of cDNAs that interact with the L1 52/55-kDa protein. (A) The structure of the mRNAs encoding the DNA polymerase (POL) and IVa2 gene products is shown. Arrows indicate cap sites, thick lines are exons, thin lines represent introns, open boxes indicate ORFs, and "A" indicates the poly(A) tail. (B) Nucleotide and amino acid sequences spanning the region of the DNA polymerase-IVa2 overlap. The 5′ and 3′ ends of the cDNAs are indicated by down arrows and up arrows, respectively. Boxed nucleotides indicate the reported cap sites of the IVa2 mRNAs (3, 22). The location of the IVa2 intron is indicated, and the locations of the 5 aa that the IVa2 ORF shares with the DNA polymerase gene (underlined) are also shown. Underlined nucleotides indicate the IVa2-E2B polyadenylation signal (2). The asterisk indicates the translation termination signal. Amino acid sequences are labeled either DNA POL or IVa2. The numbering is per van Beveren et al. (22).

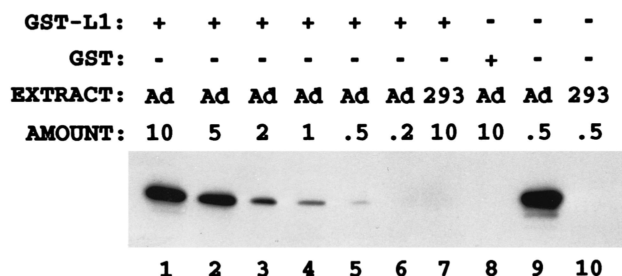

FIG. 2. The adenovirus IVa2 protein interacts with a bacterially expressed GST–52/55-kDa fusion protein. Immunoblot analysis with anti-IVa2 monoclonal antibody 1A1 to detect proteins bound to either the GST–52/55-kDa fusion protein (GST-L1) or GST is shown. EXTRACT indicates either infected (Ad) or uninfected (293) cell lysates applied to bound bacterial protein. AMOUNT indicates the amounts of extract (in microliters) applied to the bacterial protein (lanes 1 to 8) or loaded directly on the gel (lanes 9 and 10).

However, the 5′ ends of E53, L112, and L60 all map within the known IVa2 mRNA sequence, suggesting that the additional sequences at the 5′ end of the E3 clone may be a cloning artifact.

It is interesting to note that we detected IVa2-specific cDNAs in the library constructed from araC-treated 293 cells. Previously, it was reported that treatment of infected HeLa cells with hydroxyurea, which, like araC, inhibits viral and cellular DNA synthesis, blocked production of the IVa2 protein and that this block was at the level of transcription (23). In agreement with these findings, we do not detect the IVa2 protein in infected 293 cells treated with araC (11a). Possibly, the inhibition of IVa2 synthesis by araC is not at the transcriptional level. Alternatively, the block in DNA replication by araC may have been leaky, allowing some cells to replicate viral DNA and hence produce IVa2 mRNAs.

The recovery of IVa2 cDNA clones from two independent libraries, along with the high frequency with which these clones were detected, strongly indicated that this interaction was significant. In order to confirm that the 52/55-kDa protein–IVa2 interaction was not an artifact of the yeast two-hybrid system, we examined the interaction of these two proteins in vitro and in infected cells. Initially, we determined if a glutathione *S*-transferase (GST)–52/55-kDa fusion protein could interact with virally produced IVa2 protein. The 52/55-kDa ORF (aa 2 to 416) was subcloned into pGEX-3X (Pharmacia Biotech, Uppsala, Sweden) to create pGEX-L1. pGEX-L1 or pGEX-3X was induced as described previously (11). Following induction, the cells were pelleted and washed in phosphate-buffered saline (PBS) containing 0.1% β-mercaptoethanol and 2 mM EDTA. The cells were pelleted again, resuspended in PBS with β-mercaptoethanol and EDTA, lysed by sonicating them on ice five times for 10 s each time, and spun in a microcentrifuge for 5 min at 4°C. Cleared extracts were incubated for 1.5 h at 4°C with Glutathione Sepharose 4B (Pharmacia Biotech) that had been equilibrated in PBS plus 1% Triton X-100 (PBST). Bound proteins were washed twice with PBST and three times with E1A lysis buffer (13) and stored on ice until the addition of 293 lysates. Infected and mock-infected 293 cell lysates were prepared at 18 to 20 h postinfection in E1A lysis buffer as previously described (13). Cleared lysates were incubated with immobilized GST or GST–52/55-kDa protein for 1 h at 4°C, and then the lysates were subjected to three washes with E1A lysis buffer. Samples were resuspended in gel loading buffer (13), boiled, electrophoresed, transferred to Immobilon-P membrane (Millipore Corporation, Bedford, Mass.), and probed with anti-IVa2 monoclonal antibody 1A1 (17).

The immunoblot shown in Fig. 2 demonstrates that the IVa2 protein interacts specifically with the GST–52/55-kDa fusion protein (lane 1). As expected, the IVa2 protein was not detected after uninfected extracts were mixed with the GST–52/55-kDa fusion protein (Fig. 2, lane 7) or after infected cell extracts were mixed with the GST protein (lane 8). Also evident from this experiment is that not all the IVa2 protein in the cell lysate binds to the GST–52/55-kDa protein (Fig. 2; compare lanes 5 and 9). Whether this result is due to a weaker affinity of the bacterial fusion protein for IVa2 or indicates that there is a population of IVa2 that is not competent for binding to the GST–52/55-kDa protein is currently unclear. These results demonstrate that the IVa2–52/55-kDa protein interaction initially observed in the yeast two-hybrid system is also detectable with a bacterially expressed GST–52/55-kDa fusion protein in vitro.

Next, we wished to determine if the virally produced 52/55-kDa and IVa2 proteins interact during infection. To do this, we examined whether antibodies that recognize the 52/55-kDa protein could coimmunoprecipitate the IVa2 protein from infected cell extracts. Polyclonal antiserum to the 52/55-kDa protein was raised by immunizing rabbits with the purified GST–52/55-kDa fusion protein (Cocalico Biologicals Inc., Rearstown, Pa.). These antibodies were cross-linked to Affi-Gel Hz (Bio-Rad Laboratories, Hercules, Calif.) before being used. Cell lysates were incubated with antibodies bound to beads for 1 h at 4°C with rocking, and this incubation was followed by three washes with E1A lysis buffer. Then, the lysates were immunoblotted as described above (Fig. 3). Analysis of immunoprecipitated products with a mouse anti-IVa2 monoclonal antibody indicated that the IVa2 protein interacts with the 52/55-kDa protein during infection. A strong signal representing the IVa2 protein was seen only when the anti-52/55-kDa protein antiserum was used with infected cell lysates (Fig. 3, lane 3). No band was detected when uninfected cell lysates were used and when preimmune serum was used with infected cell lysates (Fig. 3, lanes 4 and 5, respectively). The specificity of the anti-52/55-kDa protein antiserum is demonstrated in Fig. 4. An immunoreactive doublet with sizes of approximately 52 and 55 kDa was detected in lysates from virally infected cells but not in uninfected cells (Fig. 4; compare lanes 1 and 4). These results demonstrate that the 52/55-kDa and IVa2 proteins interact with one another during the course of adenovirus infection.

To demonstrate independently that the IVa2 and 52/55-kDa gene products interact during viral infection, we performed the reciprocal experiment. Monoclonal anti-IVa2 immunoprecipitates were collected and immunoblotted. Figure 4 (lane 2) shows that anti-IVa2 monoclonal antibodies can coimmuno-

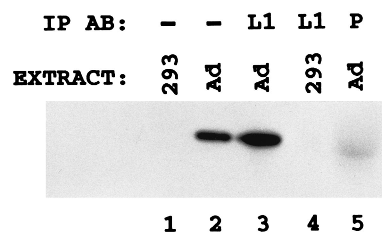

FIG. 3. Antibodies that recognize the 52/55-kDa protein can coimmunoprecipitate the IVa2 protein. Immunoblot analysis with anti-IVa2 monoclonal antibody 1A1 is shown. Lanes 3 to 5 are immunoprecipitations (IP) with either anti-52/55-kDa protein (L1) or preimmune (P) antiserum. EXTRACT indicates infected (Ad) or uninfected (293) cell lysates used for the immunoprecipitation. Lanes 1 and 2 represent analysis of whole-cell lysates from uninfected and infected cells, respectively.

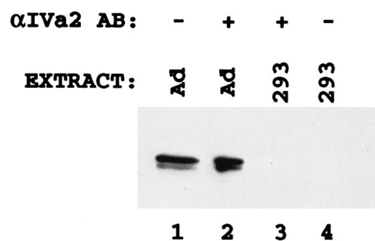

FIG. 4. Anti-IVa2 antibodies can coimmunoprecipitate the 52/55-kDa protein. Immunoblot analysis of proteins with anti-52/55-kDa protein antiserum is shown. In lanes 2 and 3, anti-IVa2 monoclonal antibody 1A1 was used to immunoprecipitate proteins from infected (Ad) and uninfected (293) cell lysates. Analysis of infected and uninfected whole-cell lysates is shown in lanes 1 and 4, respectively.

precipitate the 52/55-kDa protein from infected cell lysates. No proteins recognized by the 52/55-kDa protein antiserum were immunoprecipitated when uninfected cell lysates were used (Fig. 4, lane 3). The experiment (Fig. 4) also demonstrates that both the 52- and 55-kDa forms of the L1 protein interact with the IVa2 gene product. Further support for the specificity of the IVa2–52/55-kDa protein interaction comes from analysis of the immunoblots shown in Fig. 3 and 4 with antibodies that recognize the adenovirus type 5 72-kDa DNA-binding protein (19). Although the 72-kDa protein was abundantly detected in whole-cell lysates, it was not detected in immunoprecipitates prepared with either the IVa2 or 52/55-kDa protein antibodies (data not shown). These results strengthen the previous findings and strongly suggest that the IVa2 and 52/55-kDa proteins interact specifically during adenovirus infection.

One possible explanation for the coimmunoprecipitation results is that the 52/55-kDa protein and IVa2 antibodies cross-react with the IVa2 and 52/55-kDa proteins, respectively. However, several lines of evidence argue against this explanation. First, immunoblot analysis of whole-cell lysates with these antibodies indicates a distinct difference in band patterns (compare Fig. 3, lane 2, and Fig. 4, lane 1). Second, the anti-IVa2 monoclonal antibody does not recognize the GST–52/55-kDa fusion protein. Third, the 52/55-kDa protein was detected in lysates prepared from cells in which DNA replication had been blocked, while the IVa2 protein was not (11a). Finally, the 52/55-kDa protein was coimmunoprecipitated with three monoclonal antibodies that recognize different epitopes in the IVa2 protein (17, 17a).

Although the detection of the IVa2–52/55-kDa protein interaction in three very different systems, including the naturally occurring one, suggests that this interaction is specific and significant, a role for this interaction during the viral life cycle is currently unclear. However, in light of what is known regarding the expression patterns and functions of these two proteins, several possibilities suggest themselves. Like the 52/55-kDa protein, the IVa2 gene product has been shown to be present in assembly intermediates during adenovirus infection (14, 23). Possibly, the IVa2 and 52/55-kDa proteins interact to carry out some activity required for the assembly process. The IVa2 protein is known to specifically bind viral DNA, and perhaps the interaction of IVa2 and the 52/55-kDa protein is important for the recognition event between the viral genome and capsid or for the encapsidation process itself (17, 21). Another possibility is that the IVa2 protein is involved in the removal of the 52/55-kDa protein from assembly intermediates prior to final virion maturation. To date, we have been unable to demonstrate any difference in the 52/55-kDa protein–IVa2 interaction by coimmunoprecipitation analysis of lysates prepared from 293 cells infected with H5ts369 at either the permissive or nonpermissive temperature (data not shown). It remains possible, then, that the disruption of the 52/55-kDa protein function seen in H5ts369 is unrelated to its interaction with the IVa2 protein.

The early appearance of the 52/55-kDa protein makes it unique among the gene products transcribed from the major late promoter (MLP) and is suggestive that the protein may have functions in addition to those involved in assembly (15). Recently, the IVa2 protein has been shown to be a late-stage-specific transcriptional activator of the MLP (21). Perhaps interaction of the 52/55-kDa protein with the IVa2 gene product is required to ensure proper temporal regulation of the MLP. The IVa2 protein is a component of two complexes, DEF-A and DEF-B, that bind to and activate the MLP (17, 21). DEF-B consists of a homodimer of the IVa2 protein. The DEF-A complex has been partially purified and shown to be composed of IVa2 associated with a viral or virally induced factor (17). The 52/55-kDa protein may associate with the IVa2 protein to form the DEF-A complex. A 40-kDa peptide present in infected cells has been shown to copurify with the DEF-A complex (17). Previous immunoblot analysis of the 52/55-kDa protein has indicated that there is a major band at 40 kDa that presumably arises from the action of the viral protease at a consensus cleavage site (14). Although our analysis of IVa2 immunoprecipitates with the 52/55-kDa antiserum has not revealed a preference of the IVa2 protein for the 40-kDa peptide, it remains possible that this cleavage product is the functional partner of IVa2 in the DEF-A complex.

Temporal analysis of the transcriptional activity of the IVa2 promoter and the MLP shows that the IVa2 promoter is activated slightly before the MLP (4, 5). It is interesting to note that both the DEF-B and DEF-A complexes are required for complete activation of the MLP but that the DEF-A complex appears much earlier than the DEF-B complex (17). Perhaps the interaction of the 52/55-kDa protein and the IVa2 protein to form DEF-A prevents the premature formation of the DEF-B complex. In this scenario, full activation of the MLP could not occur until enough IVa2 has accumulated to allow formation of the homodimeric DEF-B complex. Analysis of the effects of purified 52/55-kDa protein on the DNA binding and transactivation activities of IVa2 should provide insights into the functional significance of this interaction.

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