JOURNAL OF VIROLOGY, Feb. 2005, p. 1500–1509 Vol. 79, No. 3
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.3.1500–1509.2005

Conditional Mutations in the Mitotic Chromosome Binding Function of the Bovine Papillomavirus Type 1 E2 Protein

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Received 12 July 2004/Accepted 14 September 2004

The papillomavirus E2 protein is required for viral transcriptional regulation, DNA replication and genome segregation. We have previously shown that the E2 transactivator protein and BPV1 genomes are associated with mitotic chromosomes; E2 links the genomes to cellular chromosomes to ensure efficient segregation to daughter nuclei. The transactivation domain of the E2 protein is necessary and sufficient for association of the E2 protein with mitotic chromosomes. To determine which residues of this 200-amino-acid domain are important for chromosomal interaction, E2 proteins with amino acid substitutions in each conserved residue of the transactivation domain were tested for their ability to associate with mitotic chromosomes. Chromatin binding was assessed by using immunofluorescence on both spread and directly fixed mitotic chromosomes. E2 proteins defective in the transactivation and replication functions were unable to associate with chromosomes, and those that were competent in these functions were attached to mitotic chromosomes. However, several mutated proteins that were defective for chromosomal interaction could associate with chromosomes after treatment with agents that promote protein folding or when cells were incubated at lower temperatures. These results indicate that precise folding of the E2 transactivation domain is crucial for its interaction with mitotic chromosomes and that this association can be modulated.

Papillomavirus are small DNA viruses that cause persistent epithelial lesions called papillomas (15). Papillomavirus genomes are maintained as multicopy episomes in the proliferating basal layers of papillomas; viral DNA amplification and virion particle production occur only as infected cells differentiate in the stratified epithelium. Episomal viral genomes are retained in the nucleus and are segregated between daughter cells because they are attached to condensed cellular chromosomes in mitotic cells (18, 20, 26). This interaction is mediated by the E2 transactivator protein (E2-TA), which acts as a link to tether viral DNA onto mitotic chromosomes.

In addition to its role in genome retention and segregation, the E2-TA protein regulates viral transcription and DNA replication. In bovine papillomavirus type 1 (BPV1), the E2 gene encodes three different polypeptides (22). The largest protein, expressed from the entire E2 open reading frame and designated E2-TA, is a transcriptional transactivator and is required for viral DNA replication. The two smaller E2 proteins, E2-TR and E8/E2, are encoded by the 3′ half of the E2 open reading frame and function as transcriptional repressors. The E2-TA protein contains two conserved functional domains: a N-terminal transactivation domain of ca. 200 amino acids and C-terminal DNA-binding domain and dimerization domain of ca. 100 residues. These domains are linked by a flexible hinge region. Only the E2-TA species of E2 protein is localized on mitotic chromosomes, and this interaction is mediated by the transactivation domain (6, 26).

The transactivation domain of the E2-TA protein is also critical for E2's function in DNA replication, interaction with the E1 protein, and transcriptional regulation. Deletions within this domain inactivate all functions of the E2 protein, and it appears to be extremely sensitive to disruption of conformation. The transactivation domain contains many residues that are invariant among all papillomavirus E2 sequences examined. The X-ray crystal structure of the transactivation domain of the human papillomavirus type 16 (HPV16) E2 protein was recently determined and was found to consist of an cashew-shaped domain made up of two regions (4). The N-terminal half of the domain (residues 1 to 92) contains three antiparallel α-helices and the C-terminal half (residues 110 to 210) is almost entirely antiparallel β-sheet structure. The residues between these regions form a fulcrum that orientates the domain.

The transactivation domain contains many residues that are invariant among all papillomavirus E2 sequences examined thus far. Previous studies have analyzed the transactivation, replication, and E1-binding functions of E2 proteins containing point mutations in these residues (3, 7, 10, 12, 13, 25). These studies identified amino acids that were critical for each function of the E2 protein. In the present study, we analyzed a series of BPV1 E2 proteins with point mutations in the conserved residues for their ability to interact with mitotic chromosomes. Previously, we had determined the ability of this series of E2 proteins to activate transcription, support viral DNA replication, and cooperatively bind to the origin of replication with E1 (6). Based on the results of the present study, we classified the mutated proteins into three groups. Group 1 proteins have no E2 function, and most likely have a completely defective conformation. Group 2 E2 proteins retain sig-

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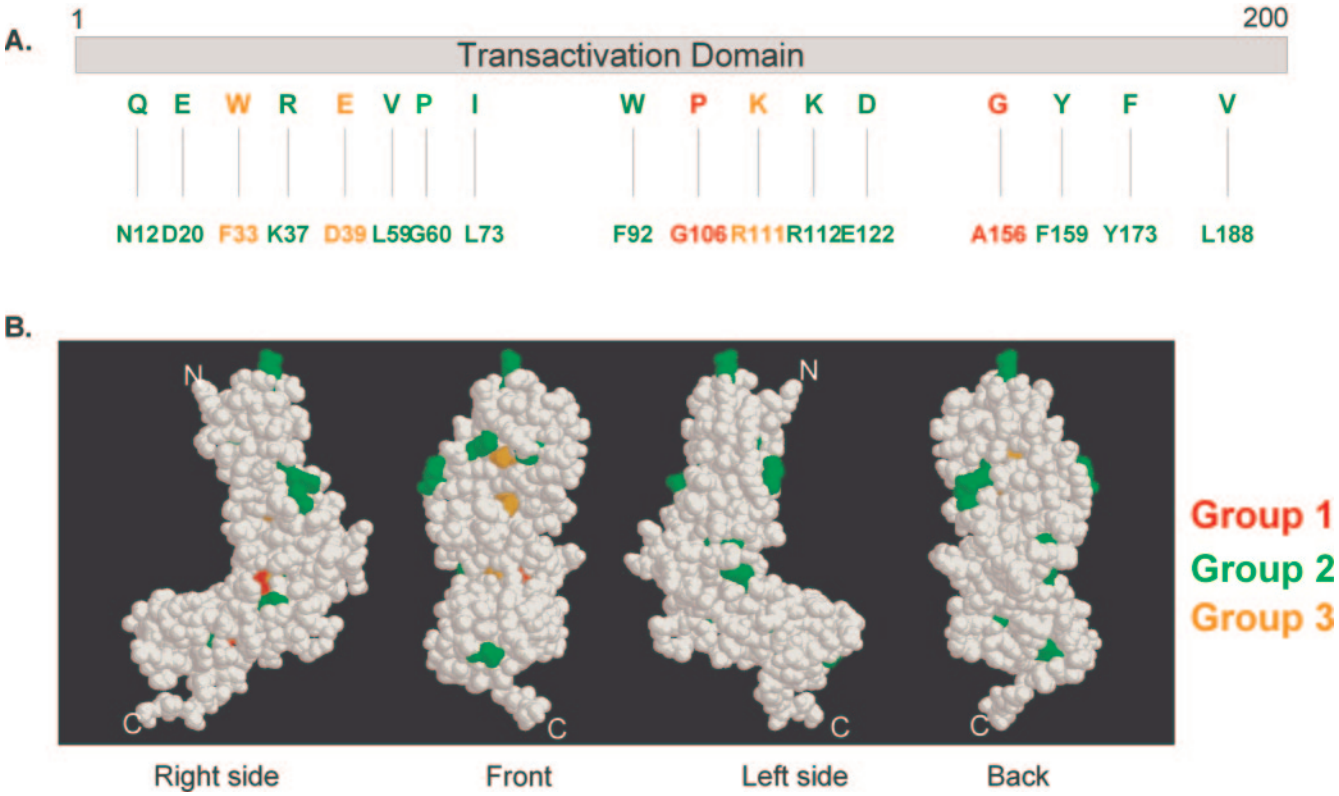


FIG. 1. (A) Amino acid substitutions in the BPV1 E2 transactivation domain. The phenotype is color coded as described for panel B. (B) The BPV1 E2 transactivation domain was homology modeled with the pdb structure of the HPV16 E2 domain (4). The mutations shown in 1A are indicated on the four views of the domain. Group 1 mutations are shown in red and are completely defective. Group 2 mutations are somewhat functional and are shown in green. The mutations shown in orange have a conditional phenotype as described in the text.

nificant transactivation and replication activity in vivo. Group 3 E2 proteins have no transactivation activity and minimal replication activity; however, they are able to cooperatively bind to the replication origin with the E1 protein in vitro, a function that is dependent on the transactivation domain (32).

We examined here the ability of these mutated E2 proteins to associate with mitotic chromosomes. Two different techniques were used to fix chromosomes for analysis of E2 binding by immunofluorescence. In one case mitotic cells grown on slides were fixed immediately and in the other mitotic chromosomes were spread by using a combination of hypotonic treatments and cytocentrifugation. The latter technique allowed visualization of numerous speckles of E2 over all mitotic chromosomes.

Analysis of the mutated proteins by using both techniques showed that, in general, chromosomal association correlated with the transactivation and replication activities of the E2 protein. However, several mutated E2 proteins that were not bound to mitotic chromosomes in directly fixed cells were observed to associate with spread mitotic chromosomes. Further analysis of these proteins showed that they had a conditional phenotype in which they could be induced to interact with mitotic chromosomes by conditions that promote protein folding and interaction. Therefore, a correctly folded transactivation domain is crucial for the interaction of E2 with mitotic chromosomes.

MATERIALS AND METHODS

Plasmids. Wild-type and mutated E2 genes were subcloned as HindIII to BamHI fragments from pTZE2$_{kz}$ plasmids containing mutated E2 genes (7) into the episomal expression vector, pMEP-4 (Invitrogen).

Cell culture. CV-1-derived cell lines were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. CV-1 cells expressing the E2 proteins were generated by transfection of pMEP-E2 plasmids by using Superfect (Qiagen), as described by the manufacturer. Hygromycin B-resistant colonies were pooled and used for all experiments.

Immunofluorescence. CV-1 cells were seeded onto glass slides (Superfrost Plus). After 24 to 48 h of growth, the cells were synchronized in S phase by the addition of 2 mM thymidine for 12 to 16 h. Thymidine was removed, and cells were cultured for 7 to 10 h before fixation. E2 expression was induced with $CdSO_4$ for the last 3 h and, in some cases, cells were blocked in mitosis by the addition of 30 ng of colchicine/ml.

For immunofluorescence using the direct-fixation method, cells were washed in phosphate-buffered saline (PBS) and fixed for 20 min in 3.2% paraformaldehyde-PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. In the hypotonic-cytospin method, cells were swollen in hypotonic buffer 1 (HB-1; 10 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 10 mM NaCl) and then in hypotonic buffer 2 (HB-2; 25% [vol/vol] PBS) for 15 min each at room temperature. The cells were further spread by centrifugation of the slides in a cytospin centrifuge at 1,500 rpm for 10 min before fixation and E2 detection. Slides were treated with RNAce-It (Stratagene) to remove RNA, and E2 was detected with a 1:10 dilution of a monoclonal antibody, B201 (provided by Elliot Androphy), and goat anti-mouse immunoglobulin G conjugated to fluorescein isothiocyanate (1:50 dilution; Jackson Immunochemicals). Slides were mounted in Vectashield (Vector Laboratories) containing 25 μg of propidium iodide/ml. Immunofluorescence was detected and captured with a Bio-Rad MRC1024 or Leica TCS-NT/SP confocal laser scanning imaging system.

Replication assay. Transient replication assays were carried out as described previously (29). In brief, 5 μg of either mutated or wild-type C59-E2 plasmid

TABLE 1. Summary of phenotypes of mutated E2 proteins

Group	Mutations	Phenotype[a]		
		Trans-activation	Repli-cation	Cooperative origin binding
1	P106G and G156A	−	−	−
2	Q12N, E20D, R37K, V59L, P60G, I73L, W92F, K112R, D122E, Y159F, F173Y, and V188L	+	+	+
3	W33F, E39D, and K111R	−	−	+

[a] The phenotype (i.e., the presence [+] or absence [−] of a particular characteristic) of each group of mutated E2 proteins, as defined previously (7), is given.

DNA, 5 μg of E1 expression plasmid (pCGEag-1235) (29), and 2 μg of replicon DNA were introduced into mouse C127 cells by electroporation. A 3.2-kb fragment of the BPV1 genome containing sequences 4786 to 7946 and 1 to 83 was used as a replicon. This fragment was cleaved from the prokaryotic vector sequences and recircularized before electroporation. At 3 and 5 days posttransfection, cells were lysed and low-molecular-weight DNA was isolated. DNA samples were digested with DpnI and HindIII, and the products were separated on a 0.8% agarose gel. The DNA fragments were transferred to Nytran (MSI; Magna Graph), probed with a fragment from the long control region (nucleotides 6958 to 6936) that was generated by PCR, and ^{32}P labeled by a random priming technique.

Transactivation assay. CV-1 cells were cotransfected with 50 ng of pMEP-E2 expression plasmid and 2.5 μg of the luciferase reporter plasmid pBS1073 by using Superfect transfection reagent (Qiagen). Lysates were prepared (cell culture lysis reagent; Promega) at 48 h posttransfection and assayed for luciferase activity by using Promega's luciferase assay system. Assays were carried out in duplicate for each sample, and the experiments were carried out at least four times.

RESULTS

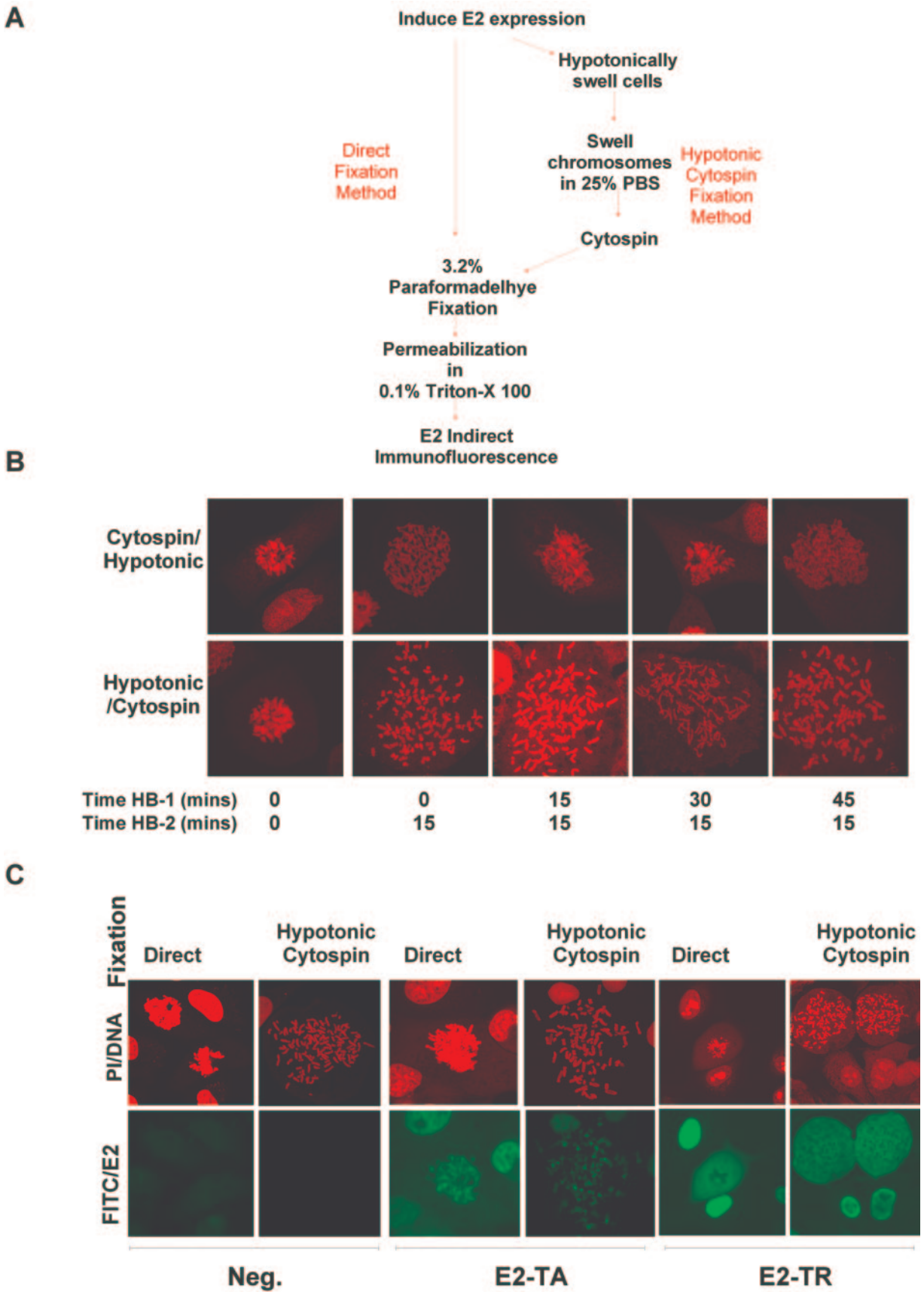
Location of mutated E2 residues on the transactivation domain. In addition to being required for the association with mitotic chromosomes, the N-terminal transactivation domain of the E2 protein is important for viral DNA replication, transcriptional transactivation, and interaction with the E1 protein. We have previously analyzed a series of E2 proteins that contain single conservative amino acid substitutions in 17 residues that are invariant among all papillomavirus E2 proteins (7) (Fig. 1A). This series of proteins were previously characterized for their ability to support viral DNA replication, activate transcription, and cooperatively bind to the origin of replication with the E1 protein. Based on their phenotypes, the proteins could be divided into three groups, as shown in Table 1. In 2000, Antson et al. determined the X-ray crystal structure of the homologous transactivation domain of the HPV16 E2 protein (4). Using the programs Look (Molecular Applications Group) and Rasmol (Roger Sayle), the structure of the BPV1 transactivation domain was modeled based on its homology with the HPV16 E2 protein (see Fig. 1B). The position of the amino acid substitutions in our series of mutated E2 proteins is indicated on the structure. The two proteins in group 1, P106G and G156A (color coded in red in Fig. 1), are defective in all E2 functions. Proline 106 and glycine 156 are located internal to the structure (4), and it is likely that their substitutions with glycine and alanine, respectively, disrupt the entire conformation of the protein. Group 2 proteins, indicated in green, retain at least partial activity in all of the functions tested. Notably, these proteins all have amino acid substitutions on the surface of the domain. Finally, group 3 contains three proteins that are completely defective for transcriptional activation and can only support viral DNA replication at very low levels. However, these proteins can still interact efficiently with the E1 protein (7). Notably, these substituted residues are located on the same face of the domain; E39D is located in the alpha-helical bundle in the N-terminal half of the domain, and W33F and K111R are found in the "fulcrum" between this and the more C-terminal β-sheet region.

E2 was expressed in CV-1 cell lines from stably maintained Epstein-Barr virus-derived episomal vectors that express E2 from an inducible metallothionein promoter (23). Analysis of E2 chromosomal association can only be carried out in cells expressing low to moderate amounts of E2; overexpression results in E2 protein that is prone to aggregation in the cytoplasm and is unable to associate with chromosomes. The E2 expression system described in the present study produces moderate levels of E2 protein that correctly localize to the nucleus and to mitotic chromosomes.

Mitotic chromosome fixation. When mitotic cells are grown on glass slides and fixed directly with 3.2% paraformaldehyde, E2 staining appears as multiple, individual fluorescent speckles on clusters of chromosomes. This method does not reveal whether the E2 protein interacts specifically with any particular region of the chromosome. Smith et al. have described a method for spreading mitotic chromosomes for immunofluorescent staining (27). In the present study, the method of Smith et al. was adapted for cells grown on slides. In brief, cells attached to slides were hypotonically swollen in buffer HB-1. Chromosomes were then further swollen in hypotonic buffer HB-2, and cells were flattened by centrifugation in a cytocentrifuge prior to fixation. A comparison of the direct-fixation and hypotonic-cytospin methods is presented in Fig. 2A. As shown in Fig. 2B, optimal spreading of chromosomes is obtained by treatment with both hypotonic buffers, followed by centrifugation to flatten the cells.

The chromosomal localization of the E2-TA (residues 1 to 410) and shorter E2-TR repressor (residues 162 to 410) proteins was analyzed by both direct-fixation and hypotonic-cytospin methods. Both methods demonstrated that the E2-TA protein is located on chromosomes (Fig. 2C), whereas the E2-TR protein is excluded, as has been shown previously (26). Furthermore, the hypotonic-cytospin method revealed that E2 was distributed in speckles along the arms of all chromosomes. This association was not obviously localized to any particular

FIG. 2. (A) Comparison of direct-fixation and hypotonic-cytospin fixation methods. (B) The effect of the order of the hypotonic treatment and cytospin centrifugation and length of hypotonic treatment on cellular mitotic chromosomes is shown. HB-1 is composed of 10 mM Tris-HCl, 10 mM NaCl, and 5 mM $MgCl_2$; HB-2 is composed of 25% PBS. (C) E2 expression was induced and cells fixed by the method indicated. E2 proteins were detected by indirect immunofluorescence with B201 E2-specific antibody. Cellular DNA was detected by propidium iodide staining (PI/DNA), and fluorescein isothiocyanate-labeled E2 protein was detected in the same field of cells (FITC/E2).


A
Induce E2 expression
Hypotonically swell cells
Swell chromosomes in 25% PBS
Direct Fixation Method
Hypotonic Cytospin Fixation Method
Cytospin
3.2% Paraformadelhye Fixation
Permeabilization in 0.1% Triton-X 100
E2 Indirect Immunofluorescence
B
Cytospin/ Hypotonic
Hypotonic /Cytospin
Time HB-1 (mins) 0 0 15 30 45
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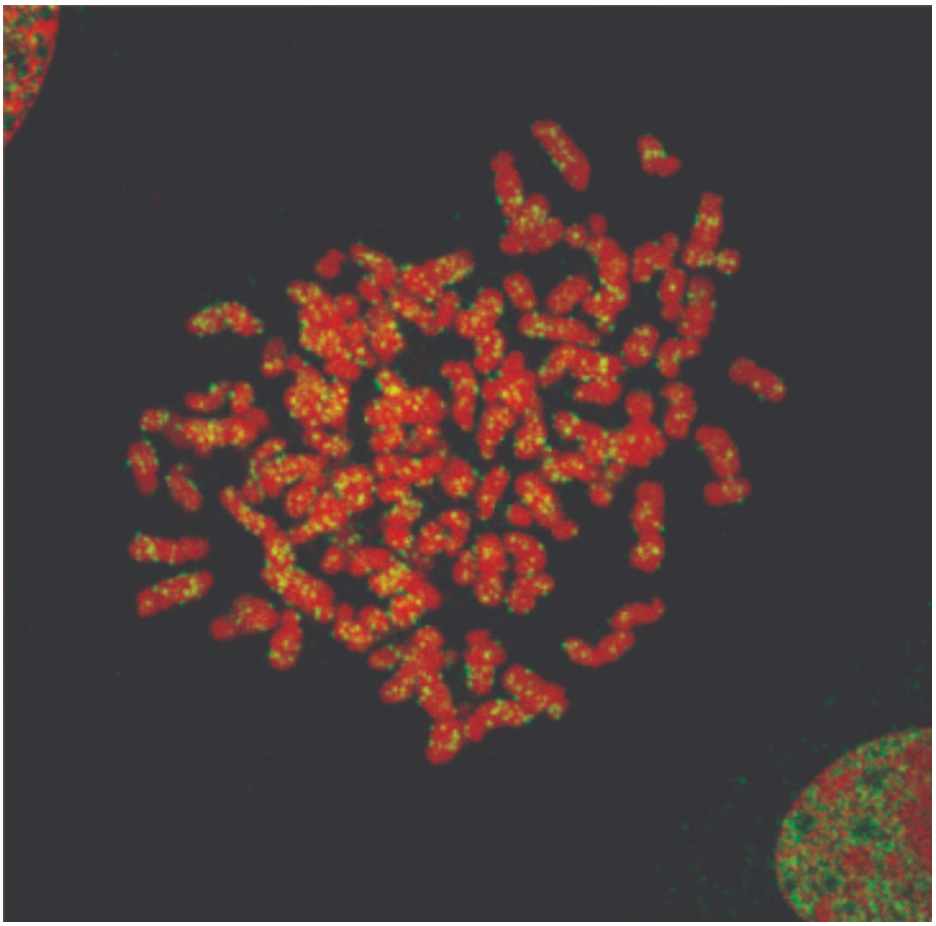

FIG. 3. E2-TA forms speckles on all mitotic chromosomes. The image shown is of the E2-TA protein (green) on mitotic chromosomes (red), as detected by the hypotonic-cytospin immunofluorescence method.

region of the chromosomes and is similar to the pattern observed for the E2 protein and BPV1 DNA by Abroi et al. (2). A more detailed image of E2-TA localization, as detected by using the Hypotonic-Cytospin method, is shown in Fig. 3.

Chromosomal association of mutated E2 proteins. The three groups of mutated E2-TA proteins were analyzed for chromosome localization by both methods (Fig. 4). The E2 proteins in group 1, which are defective for all E2 functions, were excluded from mitotic chromosomes by either method (Fig. 4A). Conversely, the E2 proteins in group 2, which are functional for transactivation and replication, were shown by either method to localize to chromosomes. Examples of three of these proteins are shown in Fig. 4B, but all proteins in this group associated with mitotic chromosomes. However, the three E2 proteins in group 3 showed a different localization with each method (Fig. 4C). With the direct-fixation method these proteins were excluded from mitotic chromosomes, but in cells fixed by the hypotonic-cytospin technique these proteins were associated with mitotic chromosomes. Further investigation showed that these proteins were induced to localize to mitotic chromosomes by the hypotonic treatment. As shown in Fig. 5A, treatment with hypotonic buffer H1 was sufficient to induce chromosomal localization of these mutants. After only 30 s of treatment, a change in the localization of E2 was observed and by 2 min these proteins were completely associated with the chromosomes. Other hypotonic buffers, such as buffer hypotonic buffer H2 or 0.075 M potassium chloride, also induced these proteins to associate with chromosomes (data not shown).

It was postulated that the conditional phenotype of the group 3 mutations was due to changes in protein conformation. Under normal conditions these proteins would be slightly unfolded and thus defective in transactivation, DNA replication, and chromosomal association. These proteins can, however, function in cooperative binding of the replication origin with the E1 protein; this could be due to the effect of E1 binding to promote the correct conformation of the E2 protein and/or due to the lower temperature conditions in this assay (i.e., room temperature). To test this hypothesis, the functions of the group 3 proteins were analyzed under conditions that promote protein folding.

After stress, heat shock proteins and chaperones help to fold and stabilize denatured proteins. Chemical chaperones can also assist in protein folding by osmotic remediation (9, 33). Naturally occurring osmolytes such as trimethylamine N-oxide control protein folding by preferentially hydrating the internally exposed backbone and side chains of partially unfolded polypeptides (5, 30) Glycerol can mimic these hydration effects

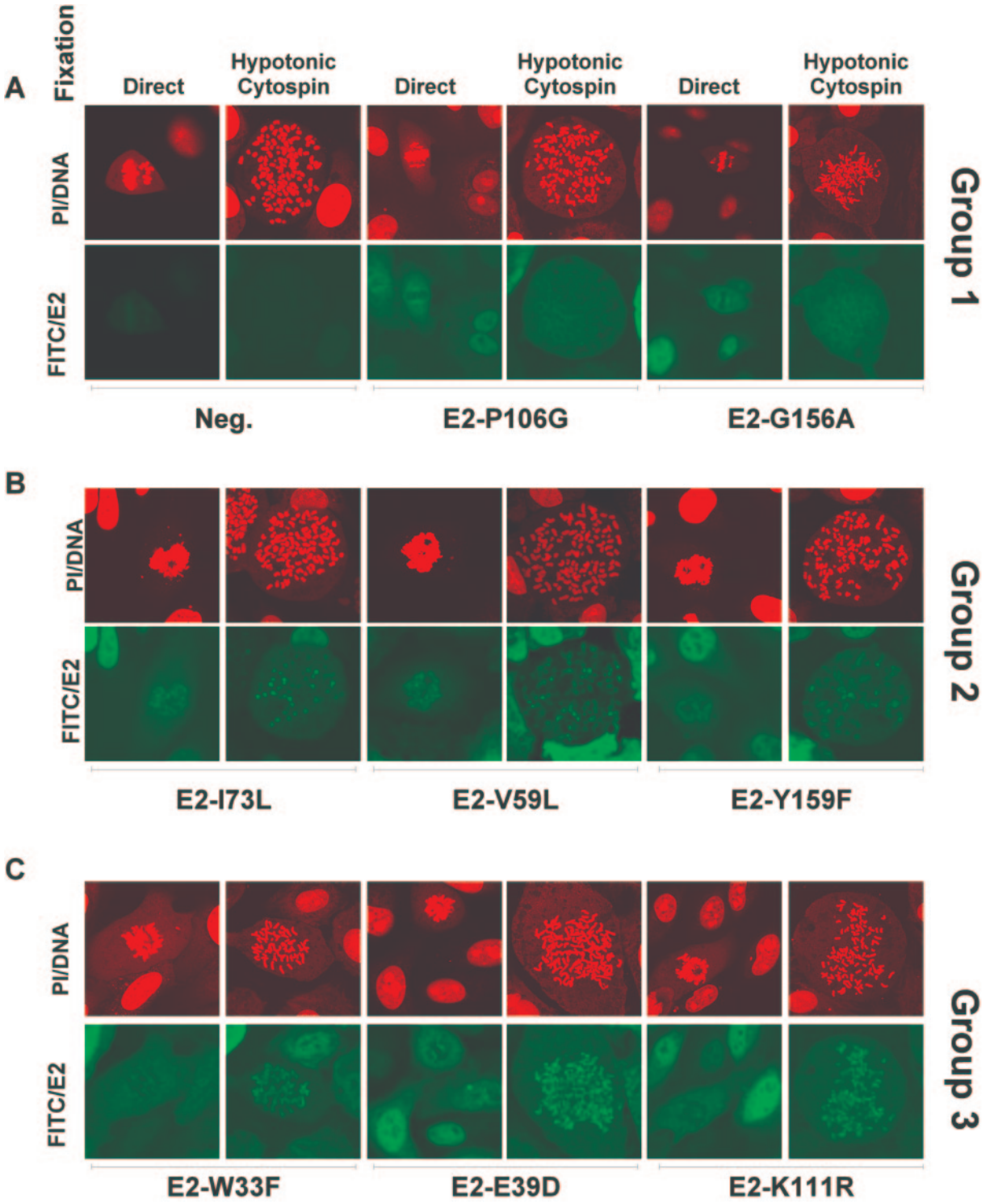

FIG. 4. Immunofluorescence was carried out as described in Fig. 2C. (A) Group 1 mutated E2 proteins are excluded from mitotic chromosomes with both fixation methods. (B) Group 2 mutated E2 proteins are associated with mitotic chromosomes with both fixation methods. (C) The association of mutated E2 proteins in group 3 with mitotic chromosomes is dependent on the fixation method.

by creating a thermodynamically unstable state due to the unfavorable entropic changes associated with the increased ordering of bound water molecules (28). Folding of the protein into its native conformation sequesters the exposed groups and excludes the osmolytes from the protein. The resulting free energy of the native conformation is much less than that of the unfolded polypeptide. Similarly, these treatments can promote protein-protein interactions. In our experiments, culture of cells in 0.5 M glycerol for 30 min before fixation resulted in chromosomal association of the W33F protein but not of the

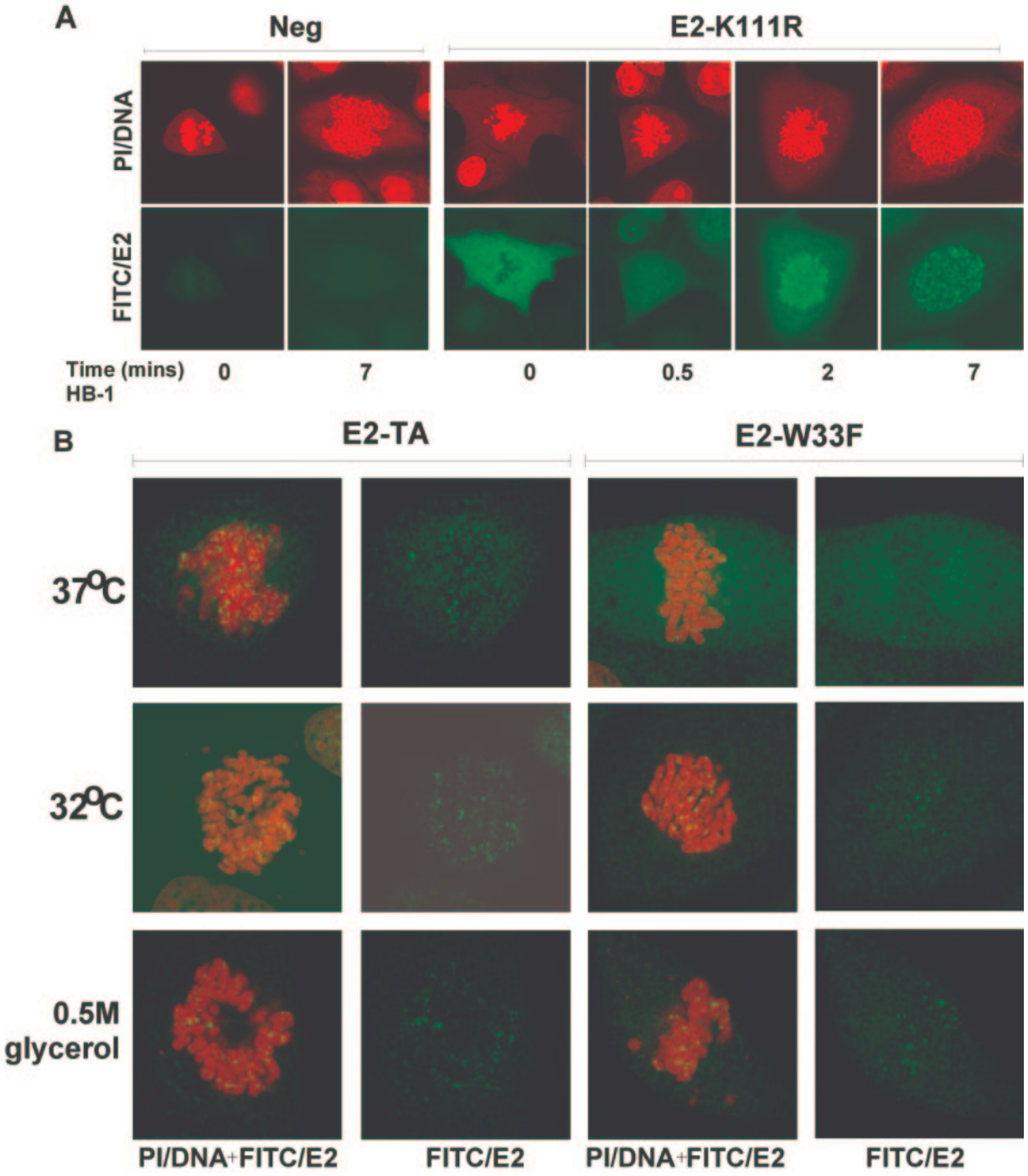

FIG. 5. (A) Hypotonic treatment induces group 3 E2 proteins to bind mitotic chromosomes. Chromosomal localization of group 3 mutated E2 proteins is related to the time of hypotonic treatment, as shown. (B) W33F E2 could be induced to bind mitotic chromosomes at 32°C or after 30 min of culture in 0.5 M glycerol.

E39D or K111R proteins (Fig. 5B). This treatment also reduced the overall levels of E2 proteins and induced a tight nuclear localization of E2 proteins that previously showed some cytoplasmic localization. Treatment with 100 mM trimethylamine N-oxide for 3 h reduced the overall levels of E2 but was unable to induce chromosomal association of any of the group 1 or 3 proteins.

Osmotic remediation has been shown to be most effective for mutated proteins that have a temperature-sensitive folding defect (8). Therefore, E2 chromosomal association was also tested when cells were cultured at lower temperatures. The W33F E2 protein (see Fig. 5B) and the K111R protein (data not shown) were able to associate with mitotic chromosomes when cells cultured at 32 or at 34°C. However, the other protein in group 3, E39D, was not rescued for mitotic chromosome binding.

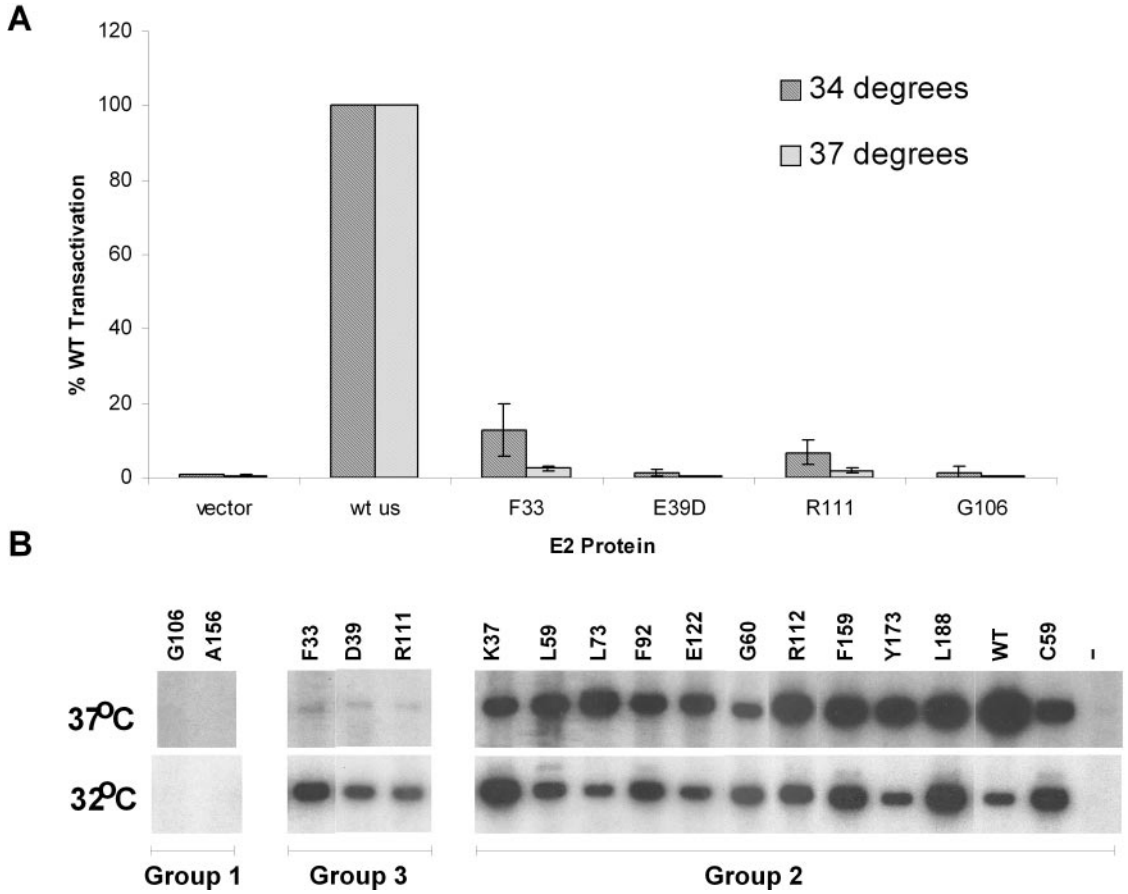

FIG. 6. (A) Group 3 E2 proteins are temperature sensitive for transactivation. CV-1 cells were electroporated with E2 expression plasmids indicated and an E2-responsive luciferase plasmid and cultured at either 34 or 37°C. Transcriptional activation is shown as a percentage of the wild-type E2 protein activity. (B) Mutated E2 proteins in group 3 are temperature sensitive for replication. Cells were electroporated with replicon DNA, E1 expression vector, and either wild-type E2 expression vector (pC59 vector), mutated E2 expression vector (in pC59 background), or empty C59 vector, as indicated, and then cultured at either 37 or 32°C. Low-molecular-weight, DpnI-resistant replicon DNA was detected by Southern blotting.

Group 3 E2 proteins were also tested for their ability to activate transcription at lower temperatures. As shown in Fig. 6A, the transactivation function of the W33F protein could be, at least partially, rescued by expression at 34°C. There was a partial rescue on the activity of the K111R protein and less so on E39D, however, which correlates with the lack of rescue of chromosome binding of E39D at lower temperatures.

The entire series of proteins was tested for their ability to support DNA replication, in concert with the E1 protein (Fig. 6B). All of the group 2 E2 proteins were able to support replication at both 32 and at 37°C. Group 1 E2 proteins were defective for replication at both temperatures. However, the group 3 proteins, which had conditional phenotypes in other assays, were only able to support DNA replication at 32°C but not at 37°C. We postulate that cooperative binding with the E1 protein in addition to lower temperatures helps promote correct folding and binding of E2 to the origin in complex with E1.

DISCUSSION

To study the interaction of E2 with specific regions of the mitotic chromosome in more detail, we developed a method to spread the mitotic chromosomes in situ that was compatible with indirect immunofluorescence for the E2 protein. This method was adapted from that of Smith et al., who used it to localize tankyrase on human telomeres (27). This technique resulted in well-spread mitotic chromosomes and demonstrated that the wild-type E2-TA was localized on individual chromosomes as speckles, whereas E2-TR was excluded from chromosomes. This technique disrupted the perichromosomally associated proteins Ki67 and topoisomerase II but did not affect the chromosomal association of phosphorylated histone H3 (data not shown). This indicates that the E2 protein is tightly bound to mitotic chromosomes. In fact, in other studies from our laboratory we find that E2-TA forms a very stable complex with mitotic chromosomes that is resistant to high salt (M. McPhillips, K. Ozato, and A. A. McBride, submitted for publication). Therefore, this immunofluorescence technique can be used to study and discriminate E2 interactions with chromosome-associated cellular proteins that mediate viral genome DNA segregation. Abroi et al. developed a technique in which cells are treated with hypotonic buffer and fixed in methanol to enable both chromosome immunofluorescence of E2 and fluorescence in situ hybridization of papillomavirus-based plasmids (2). However, in the chromosome immunofluores-

cence technique, E2 proteins that do not bind mitotic chromosomes are not detectable in the fixed mitotic cells, and additional studies are required to prove that individual E2 proteins are actually expressed in mitosis (2). The hypotonic-cytospin technique presented here will allow more precise analysis of the interaction of E2 with specific regions of mitotic chromosomes and can discriminate between tightly bound complexes of E2 with cellular chromosomal proteins.

One consideration of the hypotonic-cytospin technique, as well as other immunofluorescence methods, is that the hypotonic treatment can alter the chromosome binding activity of a small subset of mutated E2 proteins. Therefore, it is necessary to initially compare the results obtained with that of the direct-fixation technique. Surprisingly, the hypotonic treatment actually induced this subset of proteins to associate with chromosomes, and further investigation suggested that this was due to enhanced protein folding. It has been shown that hypo-osmotic stress can induce a rapid activation of heat shock transcription factor (16), but in our experiments heat shock per se did not induce chromosomal association (data not shown). Expression of E2 in cells cultured in the presence of chemical chaperones also induced certain mutated E2 proteins to associate with chromosomes. Further studies indicated that the functions of these proteins were temperature sensitive to various degrees. Together, these findings indicate that the ability of these proteins to bind chromosomes depends on correct protein folding and that their mutations do not directly prevent interaction with mitotic chromosomes.

In the present study we analyzed a series of BPV1 E2 proteins with conservative substitutions in residues that are invariant among all E2 proteins. The X-ray crystal structure of the HPV16 E2 transactivation has been determined (4), and this allowed us to model the homologous BPV1 domain and identify the positions of the mutations. All of the mutations that were localized on the surface of the domain were able to bind mitotic chromosomes and had previously been shown to be at least partially functional in other assays of E2 function (7). Therefore, it appeared that either single amino acid substitutions or the conservative nature of the substitutions were not enough to disrupt the interaction of E2 with mitotic chromosomes. Based on this finding, we have generated a new series of E2 proteins in which we have substituted patches of residues on the surface of the domain instead of individual residues (6a). In the present study, two mutations that were defective in all functions of E2, including mitotic chromosome binding, were located internally within the domain. Therefore, it is not surprising that their substitution would completely disrupt E2 structure and function.

Mutations W33F, E39D, and K111R resulted in a conditional phenotype. These residues lie on the same face of the domain, with W33 and K111 being located in the fulcrum (4). In our previous study the residues were shown to be defective for transactivation and replication but were still functional for in vitro cooperative binding to the replication origin with E1 (7). However, further analysis in the present study showed that they have a range of conditional phenotypes that depend on correct folding. All three mutations could be rescued for replication at 32°C, and all three were found to reassociate with mitotic chromosomes in the hypotonic-cytospin procedure. Only W33F and K111R, however, could be rescued for transactivation, at least partially, and mitotic chromosome binding at 34°C, indicating that they have less defective phenotypes than E39D. Mitotic chromosome binding for W33F could also be rescued by culture of cells in the chemical chaperone glycerol. W33F should prove to be a useful tool in further elucidating the mechanism of E2-mediated genome segregation. In the HPV16 E2 structure the conserved E39 residue was found to make every possible hydrogen bond through its side chain carboxyl oxygens, including one to residue Q12 (4). Therefore, it is not surprising that substitution of this residue destabilizes the protein structure. Residues and W33 and K111 are found in the fulcrum region of the protein (4), which is thought to orientate the two halves of the N-terminal domain and, again, their mutation would be predicted to subtly disrupt the structural stability of the domain.

It has now been demonstrated by many studies that the structural and conformational integrity of the transactivation domain is crucial for all of its functions. Deletion of any sequence within the domain inactivates the protein and many single amino acid substitutions are sufficient to disrupt the functions of the E2 proteins (2, 31). Several mutations in the E2 transactivation domain have been shown to result in a temperature-sensitive phenotype for replication and transactivation (11, 12). This is further supported by the present study, which indicates that subtle folding abnormalities are also sufficient to disrupt the chromosome binding function of the E2 proteins, and therefore it is unlikely that any short linear peptide sequence in the E2 N-terminal domain will be sufficient for chromosome binding. This was also indicated in a recent study by Abroi et al., who found that any deletion within the domain abolished chromosomal attachment of E2 and that mutations that interfered with binding were not in a single linear region of the E2 protein (2). The reason for this became clear when the structure was determined. The first 92 amino acids form three tightly packed alpha helices, and the C-terminal region of residues 110 to 210 is an antiparallel β-barrel structure. The intervening residues are tightly packed against both N-terminal and C-terminal regions (4). Furthermore, the structure of a major portion of the HPV18 E2 transactivation domain(14) and the structure of the HPV18 E2 transactivation domain in complex with the E1 protein (1) are virtually superimposable with that of the HPV16 E2 domain (1). Therefore, the E2 transactivation domain appears to have a single, critical conformation. Thus, the interaction of E2 with mitotic chromosomes appears to be different from that of the analogous herpesvirus proteins, EBNA1 and LANA, in which short basic peptides derived from the proteins are sufficient to mediate chromosomal attachment (17, 19, 21, 24).

The mutations in the present study were not able to separate the mitotic chromosomal binding function from that of the transactivation and replication functions. A recent study by Abroi et al. also examined the ability of a similar series of mutations for their ability to interact with mitotic chromosomes (2). These authors found that substitution of residues Q12, R37, and E90 with alanine reduces E2 chromosome attachment and that alanine substitution of E39, R68, E74, and D122 abolishes binding. Based on these results, these authors postulated that the surface of the transactivation domain containing Q12, E13, E39, and R68 comprises a region important for mitotic chromosome attachment. In our study, conservative

changes in residues Q12N, and D122E did not affect binding, and E39D could bind under certain conditions. However, it has been found that conservative amino acid changes and alanine substitutions can give very different phenotypes in the transactivation and replication functions of E2 (10, 22),

Thus, although the present study does not delineate the regions of E2 that are directly involved in interaction with mitotic chromosomes, it does indicate that the structural integrity of the domain is crucial for this interaction. Furthermore, the interaction of E2 with mitotic chromosomes can be modulated, even after mitosis has begun. These conditional E2 proteins should prove to be useful reagents in understanding the segregation function of the E2 protein.

ACKNOWLEDGMENTS

We are grateful to Maria McPhillips and Jaquelline Oliveira for critical comments on the manuscript.

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