Virology 270, 124–134 (2000)

Interaction of the Papillomavirus E2 Protein with Mitotic Chromosomes

Nathalie Bastien and Alison A. McBride[1]

Laboratory of Viral Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-0455

Received December 23, 1999; returned to author for revision January 27, 2000; accepted February 21, 2000

The bovine papillomavirus E2 transactivator protein is a multifunctional protein that activates viral transcription, cooperates in initiation of viral DNA replication, and is required for long-term episomal maintenance of viral genomes. We have shown previously that the E2 transactivator protein and bovine papillomavirus type 1 genomes are associated with mitotic chromosomes and have proposed that E2 links the genomes to cellular chromosomes to ensure segregation to daughter nuclei. In this study, we show that E2 is associated with cellular chromosomes at all stages of mitosis. We also further map the regions of E2 that are required for this association. The transactivation domain of E2 is necessary and sufficient to mediate the interaction with mitotic chromosomes; the DNA binding domain, and the flexible hinge region that separates the two domains, is not required. Furthermore, mutation of previously identified phosphorylation sites (serine residues 235, 298, and 301) has no effect on the ability of the E2 protein to bind mitotic chromosomes.

INTRODUCTION

Faithful partitioning of genetic material in cell division is essential for continued viability of all organisms. In eukaryotes, mitotic chromosomes are segregated to the daughter cells by attachment of their kinetochores to the spindle structure. The kinetochores of duplicated chromosomes are linked and move to the opposite poles of the spindle via microtubules, resulting in the delivery of one copy of each chromosome to the daughter cells. In prokaryotes, the mechanism of genome segregation is less well understood. No well-defined spindle or other structure has been identified that is responsible for directing the bacterial chromosome to the daughter cells. However, it has been shown that partitioning of low-copy number prokaryotic plasmids is mediated by the interaction of two plasmid-encoded partitioning proteins with a *cis*-acting centromere site. The newly replicated plasmids pair at mid-cell followed by separation and active movement to the poles of the predivisional cell by some unknown mechanism (Jensen and Gerdes, 1999).

DNA viruses that possess episomal genomes, such as papillomavirus or Epstein–Barr virus (EBV), must also maintain their genomes as stable extrachromosomal elements in the nuclei of infected cells. To ensure that viral genomes are maintained for generation after generation, the virus must have an efficient partitioning mechanism. We, and others, have previously reported that the E2 transactivator protein and bovine papillomavirus type-1 (BPV-1) genomes are linked to mitotic chromosomes in dividing cells (Skiadopoulos and McBride, 1998; Lehman and Botchan, 1998). We proposed a model in which E2-bound viral genomes are transiently associated with cellular chromosomes during mitosis to ensure that viral genomes are segregated to daughter cells in approximately equal numbers. Ilves *et al.* (1999) further confirmed that the E2 protein is required for the association of plasmids containing E2 DNA binding sites with mitotic chromosomes.

The E2 open reading frame (ORF) of BPV-1 encodes three different proteins. The full-length protein (E2-TA) is the transcriptional transactivator of the virus, and two smaller proteins, E2-TR and E8/E2, act as transcriptional repressors (Spalholz *et al.,* 1985; Lambert *et al.,* 1987; Choe *et al.,* 1989). The E2-TA protein is also required for viral DNA replication (Ustav and Stenlund, 1991). To initiate DNA replication, E2 binds cooperatively to the origin with the E1 protein (Mohr *et al.,* 1990; Blitz and Laimins, 1991, Wilson and Ludes-Meyers, 1991) Plasmids containing the minimal replication origin can replicate transiently in cells expressing the E1 and E2 proteins, but replicated DNA is lost with time. Long-term, stable maintenance of such plasmids requires, in addition, the minichromosome maintenance element, a *cis* element containing multiple E2 binding sites (Piirsoo *et al.,* 1996). This correlates very well with the observation that E2-TA and viral genomes are linked to cellular mitotic chromosomes, which correlates with the ability of the plasmids to be episomally maintained (Ilves *et al.,* 1999). This association also ensures that the viral genomes are enclosed in the nuclear envelope as it reforms in telophase.

Mitotic chromosomes are surrounded by a perichromosomal region that appears to consist of filaments and granules when visualized by electron microscopy (reviewed in Hernandez-Verdun and Gautier, 1994). Many

[1] To whom reprint requests should be addressed at Building 4, Room137, 4 Center Drive, MSC 0455. Fax: (301) 480-1497. E-mail: Alison_mcbride@nih.gov.

0042-6822/00 $35.00

nuclear proteins, such as those from the nucleolus and nuclear envelope, migrate to this region of the chromosome at mitosis to ensure that they will be correctly distributed to the nuclei of the daughter cells. Some of these passenger proteins interact with the chromosomes throughout mitosis and others disassociate before the nuclear membrane protein reforms in telophase. In this study, we further analyzed the stages of mitosis at which the E2-TA is found associated with cellular chromosomes.

The E2 transactivator consists of two conserved domains linked by a flexible hinge region (reviewed in McBride and Myers, 1997). The C-terminal region is required for DNA binding and dimerization, and the N-terminal domain is specifically required for the transactivation and replication functions of E2. We have previously shown that the shorter E2 repressor proteins, which do not contain the transactivation domain, cannot associate with mitotic chromosomes and that deletions in the transactivation domain abrogate the interaction (Skiadopoulos and McBride, 1998). Genetic analysis of a BPV-1 genome with mutations in the E2 phosphorylation sites led Lehman and Botchan (1998) to predict that segregation of BPV-1 genomes depends on tethering to chromosomes, which is regulated by E2 phosphorylation. They find that in cells containing this mutated viral genome, the E2-TA protein is not associated with mitotic chromosomes and the viral genomes have a segregation defect. In this study, we analyzed the role of the E2-TA phosphorylation sites on direct chromosomal attachment of E2 in the absence of the viral genome and other viral gene products and found that modification of the specific phosphorylation sites of the hinge region of E2-TA is not required. To gain more insight into the mechanism of papillomavirus genome segregation, we further determined which regions of the E2 protein are required and sufficient for interaction with mitotic chromosomes.

RESULTS

E2 expression systems

To further characterize the interaction of E2 with the mitotic chromosomes, the localization of E2 proteins was analyzed in CV-1 cells infected with recombinant SV40 viruses that encode a series of truncated E2-TA proteins or in stable lines expressing different regions of the E2-TA protein. The SV40-BPV recombinant viruses were first developed by Settleman and DiMaio (1988) and consist of an SV40 viral genome with the early region replaced with the E2-E5 region of BPV-1. These PAVA viruses express the E2 protein and replicate and are packaged in cells expressing SV40 large T antigen. Packaged recombinant virus can be used to efficiently infect the CV-1 cells used in this study. Alternatively, E2 localization was analyzed in stable CV-1 cell lines containing EBV-derived episomal vectors that expressed E2 from an inducible metallothionein promoter. In BPV-1 transformed cells and in the basal cells of a papilloma, the E2 proteins are expressed at very low levels (Hubbert *et al.,* 1988, Penrose and McBride, manuscript submitted). Analysis of E2 chromosomal association could only be carried out in cells expressing low to moderate amounts of E2; overexpression resulted in E2 that was prone to aggregate in the cytoplasm and was unable to associate with chromosomes. The two E2 expression systems described in this study produced moderate levels of E2 protein that were correctly localized to the nucleus and to mitotic chromosomes.

E2-TA remains associated with mitotic chromosomes throughout mitosis

Previous studies have documented the interaction of the E2-TA protein with mitotic chromosomes (Skiadopoulos and McBride, 1998; Lehman and Botchan, 1998; Ilves *et al.,* 1999). Many cellular proteins translocate to cellular chromosomes during mitosis to ensure that they are segregated to the nuclei of daughter cells. However, the stage of mitosis at which they associate and disassociate can vary, and many disassociate before the nuclear membrane reforms in telophase. To further analyze the temporal association of E2 with mitotic chromosomes, E2-specific immunofluorescence was carried out in a CV-1 cell line that stably expressed E2-TA from an inducible metallothionein promoter. To identify mitotic cells, the cellular DNA was labeled with Hoechst, a fluorescent dye that interchelates in DNA. Cells were also stained with an antibody directed against phosphorylated H3 histone (H3-P). H3 is phosphorylated throughout mitosis, and this modification correlates with chromatin condensation (Goto *et al.,* 1999; Hendzel *et al.,* 1997). Phosphorylation of H3 initiates late in the G_2 phase of the cell cycle; it begins in pericentromeric heterochromatin and extends throughout the condensing chromosomes as cells progress further into mitosis (Hendzel *et al.,* 1997; Wei *et al.,* 1999). Dephosphorylation of H3 begins in anaphase and is complete at telophase, just before chromatin decondensation (Hendzel *et al.,* 1997). The H3 marker allowed us to more precisely define the stage at which E2 associated with the chromosomes. Examples of cells at all stages of mitosis are shown in Fig. 1. H3 phosphorylation was initiated in late G_2 cells (no DNA condensation was yet visible with Hoechst staining) in regions of DNA described previously as pericentromeric heterochromatin (Hendzel *et al.,* 1997). Within these cells, the E2 staining occurs throughout the nucleus and colocalizes with the bulk of uncondensed chromatin and does not colocalize with H3-P. As the cells proceed to enter prophase (condensed DNA is visible), both E2 and phosphorylated histone H3 proteins are found to associate with condensed chromosomes. E2 remains associated with mitotic chromosomes at every stage of mitosis. In contrast to H3-P, E2 is still associated with condensed chromosomes in telophase and always

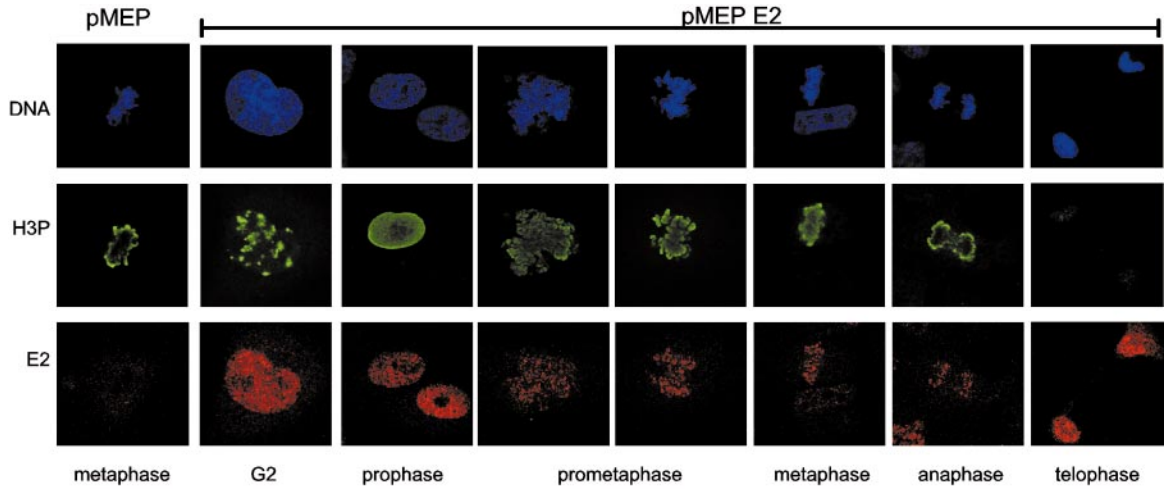

FIG. 1. Subcellular localization of E2 and phosphorylated histone H3 during the cell cycle. Immunofluorescent detection of E2 and H3-P in CV-1 pMEP-E2 cells throughout the cell cycle was analyzed with a confocal laser scanning microscope. Representative cells from each stage of mitosis are depicted: The first set of panels shows CV-1 pMEP cells that do not express E2. Anti-H3-P staining (green) is shown in the middle set of images, and anti-E2 (red) stainings are shown at the bottom. Hoechst-stained DNA images (blue) are shown along the top.

colocalizes with cellular DNA. The chromosomal association of E2 throughout mitosis most likely reflects its role in segregating viral genomes. Dissociation of the genomes before the nuclear envelope formed in telophase would be counterproductive, and many genomes could be lost in the cytoplasm. On the contrary, proteins that are required to reassemble the nucleus (such as nucleolar or nuclear membrane-derived proteins) must be released at an earlier stage.

E2 phosphorylation is not required for the association with mitotic chromosomes

The hinge region of E2 contains two major phosphorylation sites at position 298 and 301 and a minor one at position 235 (McBride *et al.,* 1989a; Lehman *et al.,* 1997). Serine residue 290 is not an actual phosphorylation site, but a mutation in this residue is present in phosphorylation defective E2 proteins that were analyzed previously (A3, A4, and AAA; McBride *et al.,* 1989a; Lehman and Botchan, 1998), and so it was analyzed in this study. Lehman and Botchan (1998) reported that in cells containing BPV-1 viral mutations that prevent E2 phosphorylation, E2 proteins are excluded from the chromosomes. To further investigate the role of E2 phosphorylation in the interaction with mitotic chromosomes, E2-TA proteins containing point mutations at the four serine residues described earlier were expressed in CV-1 cells from recombinant PAVA viruses. As shown in Fig. 2, E2-TA proteins containing a single serine-to-alanine point substitution at each of the three phosphorylation sites are closely associated with mitotic chromosomes. Similar results were obtained for the E2-TA proteins containing an alanine at position 290. E2-TA proteins containing point mutations at either both major phosphorylation sites (298, 301) as well as at all three phosphorylation sites (235, 298, 301) remain associated with mitotic chromosomes. Therefore, phosphorylation at these sites is not directly required for the association of E2 with mitotic chromatin.

The hinge of E2 is not required for the association with the mitotic chromosomes

Initial characterization of the regions of E2-TA required for association with mitotic chromosomes showed that the two repressor forms of E2, E2-TR and E8/E2, did not associate with mitotic chromosomes (Skiadopoulos and McBride, 1998). Furthermore, deletions in the N-terminal transactivation domain abrogated the interaction. The internal hinge region of E2 is thought to form a flexible link between the DNA binding domain and the transactivation domain. The length and the amino acid composition of the hinge region vary among papillomaviruses, and it is not required for the transactivation function (McBride *et al.,* 1989b). However, a small spacer region is required between the domains for the replication initiation function of E2 (Winokur and McBride, 1992). To determine whether the hinge portion of E2 is required for the association with mitotic chromosomes, the localization of E2-TA proteins containing in-frame deletions of the hinge region ($E2_{\Delta 220\text{-}283}$, $E2_{\Delta 285\text{-}309}$, $E2_{\Delta 220\text{-}309}$) was analyzed in CV-1 cells infected with recombinant SV40 virus expressing the different proteins. E2 proteins containing internal in-frame deletion that removed part of the hinge ($E2_{\Delta 220\text{-}283}$, $E2_{\Delta 285\text{-}309}$) are able to induce DNA replication and activate transcription. However, E2 proteins with deletions of the entire hinge region ($E2_{\Delta 220\text{-}309}$) are able to activate transcription and enhance E1 specific-origin binding yet are unable to support DNA replication (Winokur and McBride, 1996, 1992). As shown in Fig. 3, in mitotic cells infected with either $PAVA_{kz}E2\text{-}TA_{\Delta 220\text{-}283}$,

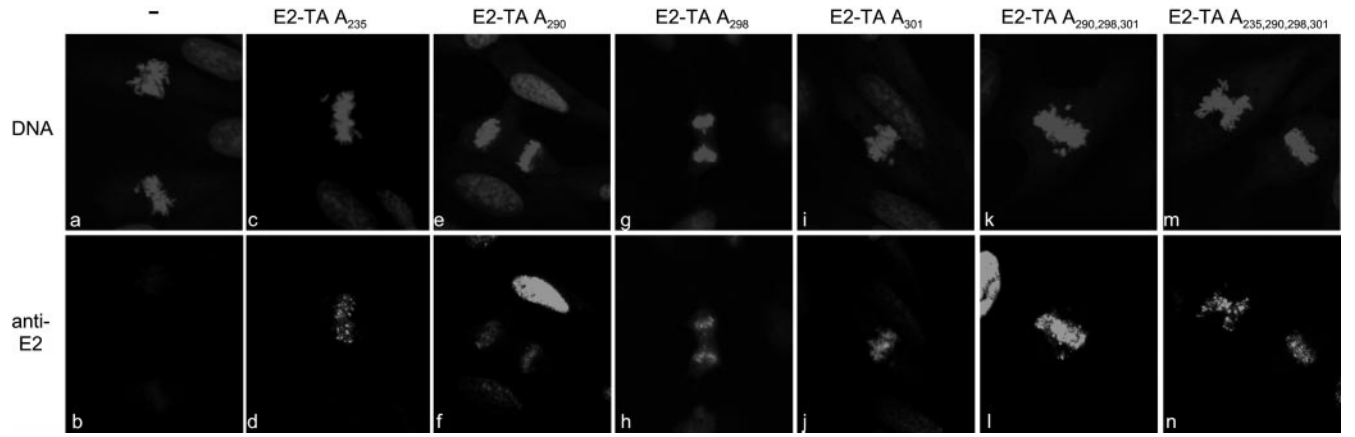

FIG. 2. E2 proteins were detected in CV-1 cells infected with recombinant PAVA viruses by immunofluorescence using the B201 E2 specific antibody. Panels a and b show uninfected CV-1 cells; panels c and d, CV-1 cells infected with $pPAVA_{kz}E2\text{-}TA\ A_{235;}$ panels e and f, infected with $pPAVA_{kz}E2\text{-}TA\ A_{290}$; panels g and h, infected with $pPAVA_{kz}E2\text{-}TA\ A_{298}$; panels i and j, infected with $pPAVA_{kz}E2\text{-}TA\ A_{301}$; panels k and l, infected with $pPAVA_{kz}E2\text{-}TA\ A_{290,\ 298,\ 301}$; and panels m and n, infected with $pPAVA_{kz}E2\text{-}TA\ A_{235,\ 290,\ 298,\ 301}$. In panels a, c, e, g, i, k, and m, cellular DNA was stained with propidium iodide to identify cells undergoing mitosis. Panels b, d, f, h, j, l, and n show FITC-labeled E2 protein in the same field of cells.

$PAVA_{kz}E2\text{-}TA_{\Delta 220\text{-}309}$, or $PAVA_{kz}E2\text{-}TA_{\Delta 285\text{-}309}$, the E2-TA proteins are observed as a random speckled pattern associated with the chromosomes that is indistinguishable from that of wild-type E2. Therefore, the hinge of E2 is not required for association with mitotic chromosomes.

The DNA binding domain of E2 is not required for the interaction with mitotic chromosome

We have previously shown that an E2-TA protein containing a point mutation (arginine to lysine at position 344) that rendered it defective in DNA binding is able to associate with mitotic chromosomes, showing that the interaction is not mediated by binding to cellular DNA sequences (Skiadopoulos and McBride, 1998). However, the DNA binding domain of E2 might still be required for interaction via another mechanism, such as interaction with other chromosomal proteins. We made several unsuccessful attempts to express the transactivation domain of E2 from recombinant SV40 virus but were never able to detect the protein by immunofluorescence. In retrospect, this was probably due to masking of the B201 antibody epitope in this protein under the conditions used for immunofluorescence. Therefore, to further examine the requirement for the DNA binding domain of E2, we generated PAVA viruses expressing E2 fusion proteins in which the E2 DNA binding domain was replaced by the Gal4 DNA binding domain. The yeast Gal4 protein is a well-characterized transcriptional transactivator that has no intrinsic replication properties in other viral systems (Bennet-Cook and Hassel, 1991; Gou and DePamphilis, 1992), and like the E2 protein, it forms dimers through the DNA binding domain. The first 147 amino acids of the GAL4 protein constitute the DNA binding and dimerization domain (Keegan *et al.,* 1986; Carey *et al.,* 1989). Three recombinant PAVA viruses were generated that expressed E2-Gal4 fusion proteins that contained the N-terminal domain of E2 and different lengths of the hinge region fused in-frame to the Gal4 DNA binding domain ($E2_{1\text{-}219}Gal4_{1\text{-}147}$, $E2_{1\text{-}250}Gal4_{1\text{-}147}$, and $E2_{1\text{-}283}Gal4_{1\text{-}147}$). These three fusion proteins are functionally active in that they are able to activate transcription from a Gal4-responsive plasmid and can enhance binding of E1 to a BPV-1 chimeric origin containing Gal4 binding sites in place of the E2 binding sites (Winokur and McBride, 1996). As shown in Fig. 4, all three E2-Gal4 fusion proteins were associated with mitotic chromosomes in CV-1-infected cells. Therefore, the specific E2 DNA binding domain is not required for the association with mitotic chromosomes. These results also confirm that the hinge region of E2 is unnecessary.

The transactivation domain of E2 is sufficient for the association with mitotic chromosomes

The E2-TR and E8/E2 repressors proteins do not associate with mitotic chromosomes, and a deletion in the transactivation domain abrogates this interaction, showing that the transactivation domain is required for this interaction(Skiadopoulos and McBride, 1998). However, these findings did not prove that the transactivation domain is sufficient for the interaction, because a DNA binding domain was always present. To determine whether the transactivation domain alone is sufficient to mediate the association with mitotic chromosomes, it was expressed in the absence of any other domain. Difficulties were encountered in detecting the E2 transactivation domain when expressed alone from recombinant PAVA viruses (see earlier). Consequently, we developed a CV-1 cell line expressing either wild-type E2 or the E2 transactivation domain ($E2_{1\text{-}218}$) fused to an SV40 nuclear localization signal and an HA (influenza hemagglutinin) epitope. The localization of E2 protein in mitotic

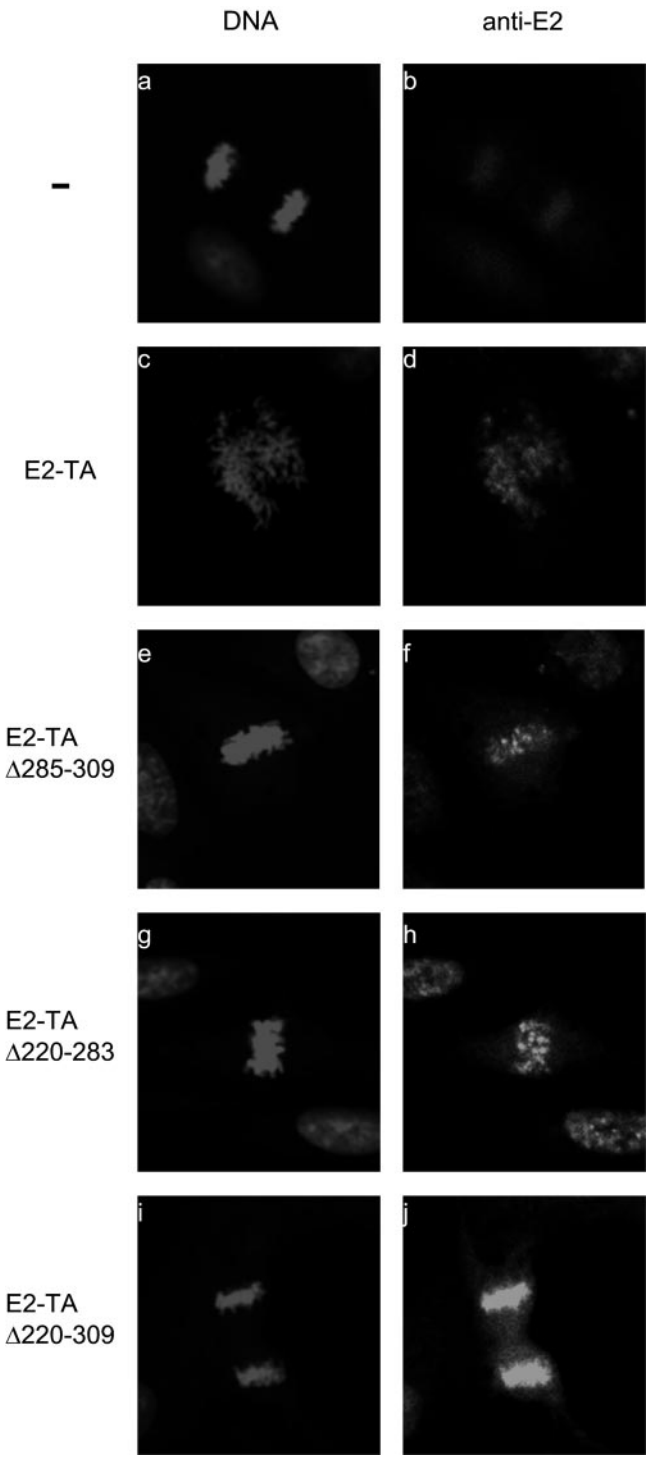

FIG. 3. E2 proteins were detected in CV-1 cells infected with recombinant PAVA viruses by immunofluorescence using the B201 E2 specific antibody. Panels a and b show uninfected CV-1 cells; panels c and d, cells infected with $pPAVA_{kz}E2\text{-}TA_{\Delta 220\text{-}283}$; panels e and f, infected with $pPAVA_{kz}E2\text{-}TA_{\Delta 285\text{-}309}$; and panels g and h, infected with $pPAVA_{kz}E2\text{-}TA_{\Delta 220\text{-}309}$. In panels a, c, e, and g, cellular DNA was stained with propidium iodide to identify cells undergoing mitosis. Panels b, d, f, and h show FITC-labeled E2 protein in the same field of cells.

cells from these lines was determined by immunofluorescence. As shown in Fig. 5, the chimeric E2 protein was found to be associated with mitotic chromosomes. Therefore, the transactivation domain is sufficient for the association with mitotic chromosomes.

DISCUSSION

We previously determined that the BPV-1 viral genomes and the E2-TA protein are associated with mitotic chromosomes (Skiadopoulos and McBride, 1998). In this study, we further characterized the regions of the E2 protein that are required for the direct association of the E2 protein with condensed chromosomes in the absence of viral genomes or other viral gene products. To do this, it was necessary to express the E2 proteins at moderate levels. When E2 was expressed at very high levels, from either transfected expression plasmids or by infection of COS-7 cells with PAVA viruses, E2 was very prone to localize in the cytoplasm and was not associated with mitotic chromosomes. We believe that under these circumstances, E2 was misfolded and aggregated. In this study, two different E2 expression systems were found to be useful: Small quantities of DNA could be delivered directly to the nucleus of CV-1 cells from recombinant PAVA viruses from which E2 was expressed from the relatively weak SV40 early promoter. Alternatively, stable CV-1 cell lines were established that contained EBV-based episomally replicating vectors that expressed E2 from an inducible metallothionein promoter (Penrose and McBride, manuscript submitted). The level of E2 expression could be regulated by the concentration of heavy metals in the medium, and at low to moderate levels of E2 protein, chromosomal association was observed.

We had shown previously that the DNA binding function of E2 is not necessary, and here we show that the DNA binding domain itself is not required for the association with mitotic chromosomes. Therefore, the interaction of E2 with mitotic chromosomes cannot be mediated by binding to cellular DNA. However, it is predicted that the E2 DNA binding domain is required to mediate the link between the E2 binding sites on the viral genomes and the cellular chromosomes. Furthermore, the flexible hinge region that separates the transactivation and DNA binding domains is not required for chromosomal interaction. An E2 protein with all of the hinge sequences removed ($E2_{\Delta 220\text{-}309}$) is able to activate transcription and enhance E1 specific-origin binding yet is unable to support DNA replication (Winokur and McBride, 1996, 1992). However, this protein efficiently associates with mitotic chromosomes, indicating that this is not the reason for its inability to support DNA replication. We also show that the transactivation domain of E2 protein is necessary and sufficient for the association with the mitotic chromosomes. This data are summarized in Fig. 6. The E2 transactivation domain is a multifunctional domain and is required to interact with several cellular factors (AMF-1, SP1, TFIIB) as well as the viral E1 and L2 proteins (Breiding *et al.,* 1997; Li *et al.,* 1991; Yao *et al.,* 1998; Benson *et al.,* 1997; Mohr *et al.,*

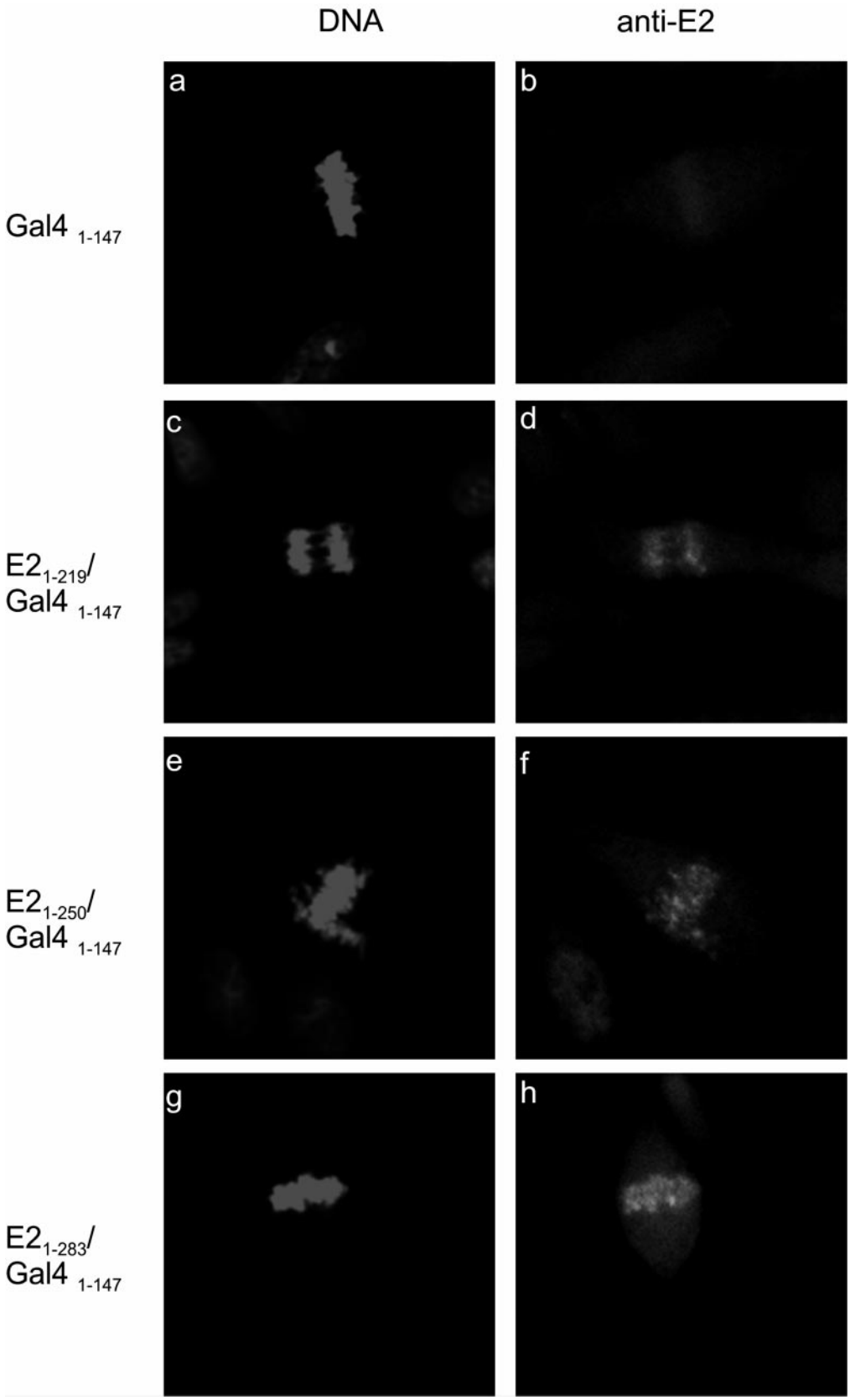

FIG. 4. E2 proteins were detected in CV-1 cells infected with recombinant PAVA viruses by immunofluorescence using the B201 E2 specific antibody. Panels a and b show CV-1 cells infected with $pPAVA_{kz}Gal4_{1-147}$; panels c and d, infected with $pPAVA_{kz}E2_{1-219}Gal4_{1-147;}$ panels e and f, infected with $pPAVA_{kz}E2_{1-250}Gal4_{1-147}$; and panels g and h, infected with $pPAVA_{kz}E2_{1-283}Gal4_{1-147}$. In panels a, c, e, and g, cellular DNA was stained with propidium iodide to identify cells undergoing mitosis. Panels b, d, f, and h show FITC-labeled E2 protein in the same field of cells.

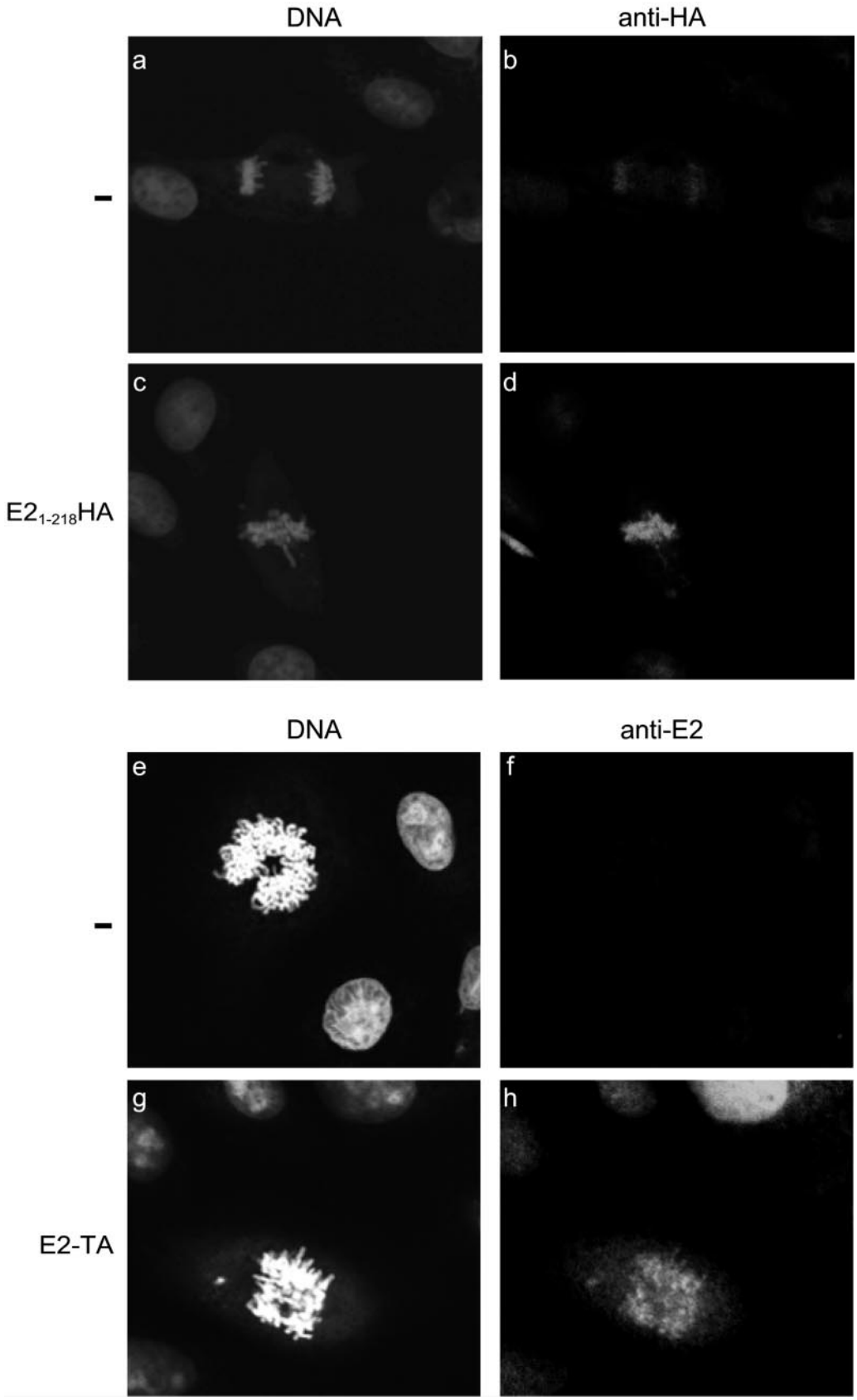

FIG. 5. E2 proteins were detected in CV-1 pMEP (a, b, c, f), pMEP-E2 (g, h); and pMEP-E2$_{1-218}$ HA cells (c, d) using either the B201 E2-specific antibody or the anti-HA epitope antibody, as indicated. In panels a, c, e, and g, cellular DNA was stained with propidium iodide to identify cells undergoing mitosis. Panels b, d, f, and h show FITC-labeled E2 protein in the same field of cells.

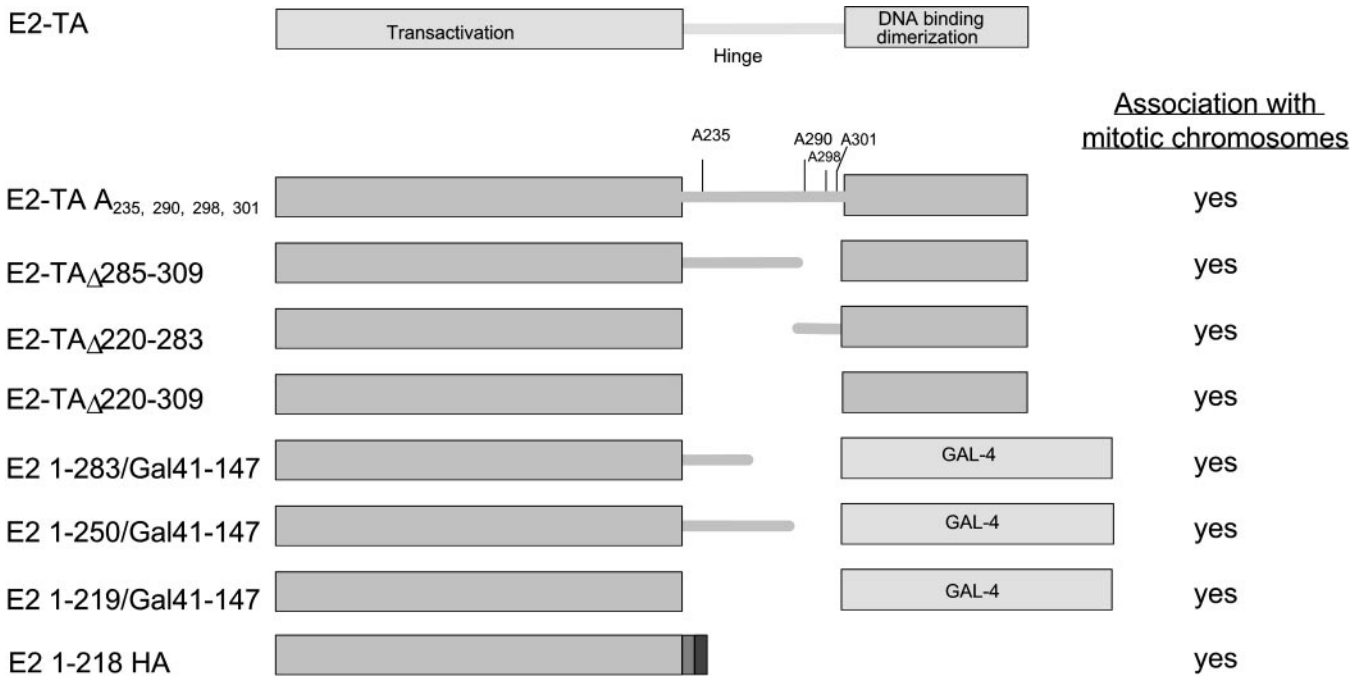

FIG. 6. Diagram of the regions of E2-TA required for interaction with mitotic chromosomes. The structures of the proteins used in this study are shown, as well as their ability to bind mitotic chromosomes. The E2 transactivator consists of two conserved domains linked by a flexible hinge region. The N-terminal domain of E2 includes approximately the first 200 amino acids and is required for the transactivation and replication functions of E2. The C-terminal 85 amino acids are required for DNA binding and dimerization. The position of the four serine residues in the hinge region that were analyzed in this study are indicated. The boxes at the C terminus of E2 1–218 HA represent the nuclear localization signal of SV40 virus and the HA epitope from influenza virus.

1990; Day *et al.,* 1998). This domain appears to have a very precise structure that is easily disrupted by deletion or point mutation. A partial structure of the HPV18 E2 transactivation domain has been solved, and it appears to have a cashew-shaped form with a glutamine-rich α-helix packed against a β-sheet framework. The determinants for replication and transcription show extensive overlap (Harris and Botchan, 1999). Additionally, we have found that E2 proteins with conserved point mutations that inactivate the transactivation function of the E2 N-terminal domain are unable to associate with mitotic chromosomes (Zheng and McBride, unpublished observations). Further studies are required to isolate the cellular protein or proteins that are responsible for the interaction of E2 with mitotic chromosomes.

E2 phosphorylation has been implicated in copy number control, as well as long-term maintenance of BPV-1 viral episomes (McBride *et al.,* 1989a; Lehman *et al.,* 1997). E2 proteins containing a serine-to-alanine mutation at position 301 (A301 E2) leads to a 10- to 20-fold increase in viral DNA copy number (McBride and Howley, 1991). Lehman and Botchan have demonstrated that viral genomes with mutations that prevent phosphorylation of the hinge region of E2 are defective for transformation and that cells containing such mutants are unable to maintain the viral genomes as episomes. Furthermore, they found that BPV-1 viral genomes and E2 protein in these cells are not associated with mitotic chromosomes, and they proposed that E2 phosphorylation regulates the association of E2 with mitotic chromosomes. Recently, we have shown that E2 proteins with a serine-to-alanine mutation in the phosphorylation site at position 301 show greatly reduced ubiquitination and degradation by the proteasome, leading to a substantial increase in half-life compared with wild-type E2 (Penrose and McBride, manuscript submitted). These findings suggest that E2 phosphorylation regulates E2 protein levels, which could in turn determine the viral genome copy number by modulating the quantity of viral genomes that are bound to chromosomes and segregated to daughter cells. However, we also find that E2 is susceptible to aggregation when overexpressed. The E2 proteins play a pivotal role in viral transcription and DNA replication, and changes in E2 levels and properties could have pleiotropic effects on many viral functions. This could explain why Lehman and Botchan observed defective segregation and E2 chromosomal association with these mutants in the background of the entire BPV-1 genome. Lehman and Botchan also found that these defects can be rescued by second site mutations in the E1 protein, indicating that the phosphorylation mutants have a complex phenotype in the background of the entire viral genome. In this study, we show that E2 proteins containing identical mutations in the phosphorylation sites are able to bind mitotic chromosomes as efficiently as wild-type E2 proteins. Therefore, in the absence of the viral genome and any other viral proteins, such as E1, phosphorylation does not appear to directly

modulate the interaction of the E2 protein with mitotic chromosomes.

Mitosis is characterized by inhibition of transcription, condensation of cellular chromosomes, and redistribution of nuclear proteins. The mitotic chromosomal periphery is composed of nonhistone proteins that are associated with the chromosomal surface during mitosis (reviewed in Hernandez-Verdun and Gautier, 1994). Many cellular "passenger proteins," such as those derived from the nuclear matrix, the nuclear membrane, and the nucleolus, migrate to the periphery of condensed mitotic chromosomes to ensure that they are equally divided to the nuclei of the daughter cells. These proteins are observed to associate and disassociate with the cellular chromosomes at different times of mitosis depending on their function within the cell.

Many chromosomal proteins associate temporally with chromosomes at specific stages of the cell cycle. For example, most transcription factors are dissociated from chromatin during mitosis (Martinez-Balbas *et al.,* 1995). The high mobility group 14/17 (HMG-14/-17) proteins form specific complexes with nucleosomes. However, during mitosis, these proteins are transiently dissociated from chromatin (Hock *et al.,* 1998). They colocalize with nuclear DNA in interphase and prophase but dissociate in metaphase and anaphase. They must be reimported into the newly formed nucleus and colocalize again with the DNA in late telophase.

Another class of chromosomal proteins associates temporally with chromosomes at specific stages of mitosis (usually metaphase and anaphase). Members of this group include many nuclear matrix and nucleolar proteins. Certain nucleolar proteins, such a fibrillarin, leave the nucleoli as they are disintegrating at the G_2/M transition and form a network around the condensing chromosomes (Fomproix *et al.,* 1998). At telophase, they move to regions of decondensing chromosomes where nucleoli are being reformed. The HIV-1 Rev protein localizes predominantly to the nucleolus of HIV-1-infected cells and behaves like a cellular nucleolar protein through mitosis until telophase. Rev begins to accumulate at chromosomal periphery in late prophase and remains associated until anaphase, when it begins to appear in numerous nucleoli-derived foci. However, when the nuclear envelope starts to reform, Rev is excluded from nuclei, and reentry is delayed until early G_1 phase (Dundr *et al.,* 1996, 1997).

Certain chromosomal proteins are similar to E2 in that they are associated with mitotic chromosomes throughout mitosis. Although most transcription factors are dissociated from chromatin at mitosis, some, such as the serum response factor $p67^{SRF}$, remain associated and are thought to be required to activate genes immediately after mitosis (Gauthier-Rouviere *et al.,* 1991,). Topoisomerase II and the cell proliferation-associated antigen Ki-67 are other examples of proteins that remain complexed to chromosomes throughout mitosis (Mo and Beck, 1999; Verheijen *et al.,* 1989). One role of E2 is to segregate viral genomes between the daughter cells and to retain them in the nucleus after cell division. To fulfill this function, the genomes must be associated with the condensed chromosomes early in mitosis and remain bound until after the nuclear envelope has been reformed. In this study, we examined the localization of E2 at each stage of mitosis and observed that, as predicted, E2 was associated with condensed chromosomes in prophase and remained visibly bound until late telophase, when the chromosomes were uncondensed. The component of mitotic chromosomes important for the association of E2 proteins is still unknown, but proteins associated with the chromosomal periphery would be good candidates.

It is now becoming apparent that many episomally replicating viruses link their genomes to mitotic chromosomes via a virally encoded protein. The EBNA1 protein of Epstein–Barr virus (Grogan *et al.,* 1983; Harris *et al.,* 1985), the immediate-early protein (IE1) of CMV (Lafemina *et al.,* 1989), and the latency-associated nuclear antigen (LANA) of human herpesvirus-8 (Ballestas *et al.,* 1999) can all associate with mitotic chromosomes. There does not seem to be any common "chromosomal association" motif in the primary sequence of these proteins, and it will be interesting to determine whether they associate with a common cellular factor on mitotic chromosomes.

MATERIALS AND METHODS

Cell culture

CV-1 and CMT4 (Gerard and Gluzman, 1985) cell lines were cultured in Dulbecco's minimal essential media supplemented with 10% fetal calf serum. Recombinant SV40 PAVA E2 viruses were produced in CMT4 cells, as described previously (Settleman and DiMaio, 1988). CV-1 cells expressing the E2 proteins from a metallothionein promoter were generated by transfection of CV-1 cells with pMEP-4 plasmids. Hygromycin B-resistant cells were selected, pooled, and screened for expression of E2 proteins by immunofluorescence.

Plasmids and viruses

A series of E2 mutations were generated in the background of a plasmid that could only express the full-length E2-TA protein; the E2-TR initiating methionine (amino acids 162 of E2-TA) in the pTZ_{kz}E2-TA constructs had been changed to an isoleucine by mutating nucleotide 3093 from ATG to ATC (Skiadopoulos and McBride, 1996). The *Dra*II–*Bst*XI E2 subfragments were subcloned from $pTZE2_{\Delta 220\text{-}283}$, $pTZE2_{\Delta 220\text{-}309}$, $pTZE2_{\Delta 285\text{-}309}$, pTZE2 $A_{290,}$ pTZE2 A_{298}, pTZE2 A_{301}, pTZE2 $A_{290,\ 298,\ 301}$, pTZE2 $A_{235,\ 290,\ 298,\ 301}$ into pTZ_{kz} E2-TA, generating pTZ_{kz}E2-$TA_{\Delta 220\text{-}283}$, pTZ_{kz}E2-$TA_{\Delta 220\text{-}309}$, pTZ_{kz}E2-$TA_{\Delta 285\text{-}309}$, pTZ_{kz}E2-TA $A_{290,}$ pTZ_{kz}E2-TA A_{298}, pTZ_{kz}E2-TA A_{301}, pTZ_{kz}E2-TA $A_{290,\ 298,\ 301}$, and

pTZ_{kz}E2-TA $A_{235,\ 290,\ 298,\ 301}$. *Bst*EII–*Bst*XI fragments containing the entire E2 ORF were subcloned from these TZ_{kz}E2-TA plasmids into $pPAVA_{kz}$E2-TA (Skiadopoulos and McBride, 1996) to generate $pPAVA_{kz}E2\text{-}TA_{\Delta 220\text{-}283}$, $pPAVA_{kz}E2\text{-}TA_{\Delta 220\text{-}309}$, $pPAVA_{kz}E2\text{-}TA_{\Delta 285\text{-}309}$, $pPAVA_{kz}$E2-TA A_{290}, $pPAVA_{kz}$E2-TA A_{298}, $pPAVA_{kz}$E2-TA A_{301}, $pPAVA_{kz}$E2-TA $A_{290,\ 298301}$, and $pPAVA_{kz}$E2-TA $A_{235,\ 290,\ 298,\ 301}$. pTZ_{kz}E2-TA A_{235} was generated by subcloning an E2 *Dra*II–*Bst*XI fragment from C59 into pTZ_{kz}E2-TA. C59 is a cDNA cloned from a bovine wart library (Yang *et al.,* 1985). The E2 expressed from this cDNA has a serine-to-alanine mutation at position 235; it is not clear whether this is a mutation generated during cloning or a natural variant of BPV-1. The *Bst*EII–*Bst*XI E2 fragment was subcloned from pTZ_{kz}E2-TA A_{235} into $pPAVA_{kz}$E2-TA to generate $pPAVA_{kz}$E2-TA A_{235}. A series of C59-based plasmids expressing chimeric proteins containing the E2 transactivation domain and portions of the hinge region linked in-frame to the Gal4 DNA binding domain have been described previously (Winokur and McBride, 1996). A *Bst*EII–*Bst*XI fragment was subcloned from these plasmids into $pPAVA_{kz}$E2-TA to generate $pPAVA_{kz}E2\text{-}TA_{1\text{-}219}Gal4_{1\text{-}147}$, $pPAVA_{kz}E2\text{-}TA_{1\text{-}250}Gal4_{1\text{-}147}$, $pPAVA_{kz}E2\text{-}TA_{1\text{-}283}Gal4_{1\text{-}147}$, and pPAVA-$Gal4_{1\text{-}147}$. A plasmid that expresses the N-terminal domain of E2 linked to the SV40 nuclear localization signal (PKKKRKV) and the influenza HA epitope (residue 98–106 of the HA protein: YPYDVPDYA) was generated by inserting double-stranded oligonucleotides that encoded these signals (AM520: GCTAGCTACCCATACGACGTCCCAGACTACGCTTGAG and AM521 GATCCTCAAGCGTAGTCTGGGACGTCGTATGGGTA) at the *Dra*I site of pTZ_{kz}E2 to generate $pTZ_{kz}E2_{1\text{-}218}$HA. An EBV-based episomal vector that expresses E2 from an inducible metallothionein promoter was generated by cloning the *Hin*dIII–*Bam*HI fragment of $pTZkzE2_{1\text{-}218}$ HA into pMEP-4 (InVitrogen, San Diego, CA) generating $pMEP\text{-}E2_{1\text{-}218}$HA. pMEP-E2, expressing the full-length E2 protein, will be described elsewhere (Penrose and McBride, manuscript submitted).

Immunofluorescence

Cells were plated onto glass slides 16 h before infection with PAVA viruses or induction of the metallothionein promoter. For PAVA virus E2 expression, CV-1 cells were infected with virus at a high multiplicity of infection and were analyzed for E2 expression after 36–40 h. For induction of wild-type E2-TA expression from CV-1 cells stably transfected with the pMEP plasmids, cells were induced by the addition of 1 μM $CdSO_4$ for 4.5 h; for induction of $E2\text{-}TA_{1\text{-}218}$NLS, HA cells were induced with 1.5 μM $CdSO_4$ for 30 h. Cells were fixed for 20 min in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and blocked in 0.25% gelatin, 0.25% BSA in PBS. Mouse monoclonal antibody B201 (provided by Elliot Androphy) was added at a dilution of 1:10. Antiserum against the HA epitope was obtained from Boehringer-Mannheim (rat monoclonal, clone 3F10; Indianapolis, IN) and used at a concentration of 5 μg/ml. Antiserum against phosphorylated histone H3 was obtained from Upstate Biotechnology (rabbit polyclonal; Lake Placid, NY) and used at 5 μg/ml. Slides were incubated with the primary antibodies, washed with PBS, and incubated with either goat anti-mouse IgG (conjugated to fluorescein isothiocyanate or Cy5) or donkey anti-rat IgG (conjugated to fluorescein isothiocyanate) or donkey anti-rabbit IgG (conjugated to Oregon green, 1:100; Jackson Immunoresearch, West Grove, PA). Where indicated, cellular DNA was stained for 5 min with Hoechst 33258 (5 μg/ml). After washes in PBS, slides were mounted in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Where stated, 25 μg/ml propidium iodide was added to the mounting solution. Fluorescence was detected and photographed with Bio-Rad (Hercules, CA) MRC1024 and Leica TCS confocal laser scanning imaging systems.

ACKNOWLEDGMENTS

We are grateful to Elliot Androphy for providing the B201 monoclonal antibody and to David Allis for suggesting the use of the antiserum against phosphorylated H3. We also thank Carl Baker and Kerri Penrose for critical comments on the manuscript.

REFERENCES

Ballestas, M. E., Chatis, P. A., and Kaye, K. M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* **284**, 641–644.

Bennet-Cook, E. R., and Hassel, J. A. (1991). Activation of polyomavirus DNA replication by yeast GAL4 is dependent on its transcriptional activation domains. *EMBO J.* **10,** 959–969.

Benson, J. D., Lawande, R., and Howley, P. M. (1997). Conserved interaction of the papillomavirus E2 transcriptional activator proteins with human and yeast TFIIB proteins. *J. Virol.* **71,** 8041–8047.

Blitz, I. L., and Laimins, L. A. (1991). The 68-kilodalton E1 protein of bovine papillomavirus is a DNA binding phosphoprotein which associates with the E2 transcriptional activator in vitro. *J. Virol.* **65,** 649–656.

Breiding, D. E., Sverdrup, F., Grossel, M. J., Moscufo, N., Boonchai, W., and Androphy, E. J. (1997). Functional interaction of a novel cellular protein with the papillomavirus E2 transactivation domain. *Mol. Cell. Biol.* **17,** 7208–7219.

Carey, M., Kakidani, H., Leatherwood, J., Mostashari, F., and Ptashne, M. (1989). An amino-terminal fragment of GAL4 binds DNA as a dimer. *J. Mol. Biol.* **209,** 423–432.

Choe, J., Vaillancourt, P., Stenlund, A., and Botchan, M. (1989). Bovine papillomavirus type 1 encodes two forms of a transcriptional repressor: Structural and functional analysis of new viral cDNAs. *J. Virol.* **63,** 1743–1755.

Day, P. M., Roden, R. B., Lowy, D. R., and Schiller, J. T. (1998). The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. *J. Virol.* **72,** 142–150.

Dundr, M., Meier, U. T., Lewis, N., Rekosh, D., Hammarskjold, M. L., and Olson, M. O. (1997). A class of nonribosomal nucleolar components is located in chromosome periphery and in nucleolus-derived foci during anaphase and telophase. *Chromosoma* **105,** 407–417.

Dundr, M., Leno, G. H., Lewis, N., Rekosh, D., Hammarskjoid, M. L., and Olson, M. O. (1996). Location of the HIV-1 Rev protein during mitosis:

inactivation of the nuclear export signal alters the pathway for post-mitotic reentry into nucleoli. *J. Cell Sci.* **109,** 2239–2251.

Fomproix, N., Gebrane-Younes, J., and Hernandez-Verdun, D. (1998). Effects of anti-fibrillarin antibodies on building of functional nucleoli at the end of mitosis. *J. Cell Sci.* **111,** 359–372.

Gauthier-Rouviere, C., Cavadore, J. C., Blanchard, J. M., Lamb, N. J., and Fernandez, A. (1991). p67SRF is a constitutive nuclear protein implicated in the modulation of genes required throughout the G1 period. *Cell Regul.* **2,** 575–588.

Gerard, R. D., and Gluzman, Y. (1985). New host cell system for regulated simian virus 40 DNA replication. *Mol. Cell. Biol.* **5,** 3231–3240.

Goto, H., Tomono, Y., Ajiro, K., Kosako, H., Fujita, M., Sakurai, M., Okawa, K., Iwamatsu, A., Okigaki, T., Takahashi, T., and Inagaki, M. (1999). Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation. *J. Biol. Chem.* **274,** 25543–25549.

Gou, Z.-S., and DePamphilis, M. L. (1992). Specific transcription factors stimulate SV40 and polyomavirus origins of DNA replication. *Mol. Cell. Biol.* **12,** 2514–2524.

Grogan, E. A., Summers, W. P., Dowling, S., Shedd, D., Gradoville, L., and Miller, G. (1983). Two Epstein-Barr viral nuclear neoantigens distinguished by gene transfer, serology, and chromosome binding. *Proc. Natl. Acad. Sci. USA* **80,** 7650–7653.

Harris, A., Young, B. D., and Griffin, B. E. (1985). Random association of Epstein-Barr virus genomes with host cell metaphase chromosomes in Burkitt's lymphoma-derived cell lines. *J. Virol.* **56,** 328–332.

Harris, S. F., and Botchan, M. R. (1999). Crystal structure of the human papillomavirus type 18 E2 activation domain. *Science* **284,** 1673–1677.

Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P., and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106,** 348–360.

Hernandez-Verdun, D., and Gautier, T. (1994). The chromosome periphery during mitosis. *Bioessays* **16,** 179–185.

Hock, R., Scheer, U., and Bustin, M. (1998). Chromosomal proteins HMG-14 and HMG-17 are released from mitotic chromosomes and imported into the nucleus by active transport. *J. Cell Biol.* **143,** 1427–1436.

Ilves, I., Kivi, S., and Ustav, M. (1999). Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which Is mediated by the viral E2 protein and its binding sites. *J. Virol.* **73,** 4404–4412.

Jensen, R. B., and Gerdes, K. (1999). Mechanism of DNA segregation in prokaryotes: ParM partitioning protein of plasmid R1 co-localizes with its replicon during the cell cycle. *EMBO J.* **18,** 4076–4084.

Keegan, L., Gill, G., and Ptashne, M. (1986). Separation of DNA binding from the transcription-activating function of a eukaryotic transcriptional activator protein. *Science* **231,** 699–704.

Lafemina, R. L., Pizzorno, M. C., Mosca, J. D., and Hayward, G. S. (1989). Expression of the acidic nuclear immediate-early protein (IE1) of human cytomegalovirus in stable cell lines and its preferential association with metaphase chromosomes. *Virology* **172,** 584–600.

Lambert, P. F., Spalholz, B. A., and Howley, P. M. (1987). A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. *Cell* **50,** 69–78.

Lehman, C. W., and Botchan, M. R. (1998). Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation. *Proc. Natl. Acad. Sci. USA* **95,** 4338–4343.

Lehman, C. W., King, D. S., and Botchan, M. R. (1997). A papillomavirus E2 phosphorylation mutant exhibits normal transient replication and transcription but is defective in transformation and plasmid retention. *J. Virol.* **71,** 3652–3665.

Li, R., Knight, J. D., Jackson, S. P., Tjian, R., and Botchan, M. R. (1991). Direct interaction between Sp1 and the BPV enhancer E2 protein mediates synergistic activation of transcription. *Cell* **65,** 493–505.

Martinez-Balbas, M. A., Dey, A., Rabindran, S. K., Ozato, K., and Wu, C. (1995). Displacement of sequence-specific transcription factors from mitotic chromatin. *Cell* **83,** 29–38.

McBride, A. A., Bolen, J. B., and Howley, P. M. (1989a). Phosphorylation sites of the E2 transcriptional regulatory proteins of bovine papillomavirus type 1. *J. Virol.* **63,** 5076–5085.

McBride, A. A., Byrne, J. C., and Howley, P. M. (1989b). E2 polypeptides encoded by bovine papillomavirus type 1 form dimers through the common carboxyl-terminal domain: Transactivation is mediated by the conserved amino-terminal domain. *Proc. Natl. Acad. Sci. USA* **86,** 510–514.

McBride, A. A., and Howley, P. M. (1991). Bovine papillomavirus with a mutation in the E2 serine 301 phosphorylation site replicates at a high copy number. *J. Virol.* **65,** 6528–6534.

McBride, A. A., and Myers, G. (1997). The E2 proteins: An update. *In* "Human Papillomaviruses 1997" (G. Myers, C. Baker, K. Munger, F. Sverdrup, A. McBride, and H.-U. Bernard, Eds.), Los Alamos National Laboratory, Los Alamos, NM.

Mo, Y. Y., and Beck, W. T. (1999). Association of human DNA topoisomerase IIalpha with mitotic chromosomes in mammalian cells is independent of its catalytic activity. *Exp. Cell Res.* **252,** 50–62.

Mohr, I. J., Clark, R., Sun, S., Androphy, E. J., MacPherson, P., and Botchan, M. R. (1990). Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science* **250,** 1694–1699.

Piirsoo, M., Ustav, E., Mandel, T., Stenlund, A., and Ustav, M. (1996). Cis and trans requirements for stable episomal maintenance of the BPV-1 replicator. *EMBO J.* **15,** 1–11.

Settleman, J., and DiMaio, D. (1988). Efficient transactivation and morphologic transformation by bovine papillomavirus genes expressed from a bovine papillomavirus/simian virus 40 recombinant virus. *Proc. Natl. Acad. Sci. USA* **85,** 9007–9011.

Skiadopoulos, M. H., and McBride, A. A. (1996). The BPV-1 E2 transactivator and E2-TR repressor use different nuclear localization signals. *J. Virol.* **70,** 1117–1124.

Skiadopoulos, M. H., and McBride, A. A. (1998). BPV1 viral genomes and the E2 transactivator protein are associated with cellular metaphase chromosomes. *J. Virol.* **72,** 2079–2088.

Spalholz, B. A., Yang, Y. C., and Howley, P. M. (1985). Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. *Cell* **42,** 183–191.

Ustav, M., and Stenlund, A. (1991). Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. *EMBO J.* **10,** 449–457.

Verheijen, R., Kuijpers, H. J., van Driel, R., Beck, J. L., van Dierendonck, J. H., Brakenhoff, G. J., and Ramaekers, F. C. (1989). Ki-67 detects a nuclear matrix-associated proliferation-related antigen. II. Localization in mitotic cells and association with chromosomes. *J. Cell Sci.* **92,** 531–540.

Wei, Y., Yu, L., Bowen, J., Gorovsky, M. A., and Allis, C. D. (1999). Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. *Cell* **97,** 99–109.

Wilson, V. G., and Ludes-Meyers, J. (1991). A bovine papillomavirus E1-related protein binds specifically to bovine papillomavirus DNA. *J. Virol.* **65,** 5314–5322.

Winokur, P. L., and McBride, A. A. (1992). Separation of the transcriptional activation and replication functions of the bovine papillomavirus-1 E2 protein. *EMBO J.* **11,** 4111–4118.

Winokur, P. L., and McBride, A. A. (1996). The transactivation and DNA binding domains of the BPV-1 E2 protein have different roles in cooperative origin binding with the E1 protein. *Virology* **221,** 44–53.

Yang, Y. C., Okayama, H., and Howley, P. M. (1985). Bovine papillomavirus contains multiple transforming genes. *Proc. Natl. Acad. Sci. USA* **82,** 1030–1034.

Yao, J. M., Breiding, D. E., and Androphy, E. J. (1998). Functional interaction of the bovine papillomavirus E2 transactivation domain with TFIIB. *J. Virol.* **72,** 1013–1019.