The Brn-3a POU family transcription factor has previously been shown to activate the human papilloma virus type 16 (HPV-16) promoter driving the expression of the E6- and E7-transforming proteins. Moreover, Brn-3a is overexpressed approximately 300-fold in cervical biopsies from women with cervical intra-epithelial neoplasia type 3 (CIN3) compared with normal cervical material. To test the role of Brn-3a in cervical neoplasia we have manipulated its expression in cervical carcinoma-derived cell lines with or without endogenous HPV genes. In HPV-expressing cells, reduction in Brn-3a expression specifically reduces HPV gene expression, growth rate, saturation density and anchorage-independent growth, whereas these effects are not observed when Brn-3a expression is reduced in cervical cells lacking HPV genomes. Together with our previous observations, these findings indicate a critical role for Brn-3a in regulating HPV gene expression and thereby in controlling the growth/transformation of cervical cells.

It is now generally accepted that the oncogenic human papilloma viruses types 16 and 18 (HPV-16 and HPV-18) play a key role in cervical cancer. Thus these viruses encode specific transforming proteins (E6, E7) and are found in the great majority of women with cervical cancer (1–3). Despite this, however, both HPV-16 and HPV-18 can be detected in women with undetectable or minimal cervical abnormality (4, 5). It is therefore unclear what factors trigger HPV-16 or HPV-18 to initiate the transformation of cervical cells to a malignant phenotype. It has recently been shown that one potential mechanism for regulating HPV-induced transformation is the regulation of the activity of the transforming proteins E6 and E7. Thus, a natural polymorphism exists in the human population with some individuals having a p53 tumor suppressor protein with a proline residue at position 72, whereas others have an arginine at this position (6). As p53 with an arginine residue is more efficiently degraded by the HPV E6 protein, individuals with this amino acid have a higher risk of cervical cancer caused by HPV (6).

Evidently, another potential mechanism for regulating transformation by HPV would be the control of the production of the E6 and E7 proteins by cellular regulatory factors. Thus, it has been shown that the upstream regulatory region (URR) of the virus genome, which controls the expression of the genes encoding E6 and E7, is specifically active in cervical cells (7, 8), and it has therefore been suggested that the regulation of HPV URR activity by cellular transcription factors may play a key role in regulating viral transforming activity in different cell types and different individuals (9). However, although numerous studies have documented binding of several cellular transcription factors to the URR (10–13), these factors are generally ubiquitously expressed and may not therefore fully explain the cervical cell-specific activity of the URR or its activation leading to transformation in vivo.

In previous studies, we showed that a sequence ATGCAATT in the region of the URR that confers cervical specific activity was able to stimulate URR activity in cervical cell lines while inhibiting its activity in noncervical cells (14). Thus, this motif binds the ubiquitously expressed POU family transcription factor Oct-1, which represses its activity in noncervical cells (14, 15). Conversely, in cervical cells this motif can, in addition, bind the POU family transcription factors Brn-3a and Brn-3b, which were originally identified in neuronal cells (16–18) but that are also expressed in cervical cells but not by other cell types (17). Brn-3a (also known as Brn-3.0 (16, 17)) and Brn-3b (also known as Brn-3.2 (17, 18)) are members of the POU family of transcription factors (for reviews see Refs. 19 and 20), which are closely related to one another but are encoded by distinct genes (21). Most interestingly, Brn-3a activates transcription directed by the HPV URR, and this effect is dependent on the ATGCAATT motif (22). Conversely, Brn-3b represses such transcription via the ATGCAATT motif and interferes with activation by Brn-3a (22).

In view of the possibility that the balance between Brn-3a and Brn-3b expression might play a key role in determining the activity of the HPV URR and therefore the progression of cervical cancer, we previously examined the expression of these factors in cervical biopsies from women with no detectable cervical abnormality and those with cervical intra-epithelial neoplasia grade 3 (CIN3) lesions. Most interestingly, although the level of Brn-3b expression was similar in the two groups, the mean level of Brn-3b was elevated more than 30-fold in the samples from women with CIN3 compared with the normal samples (23).

Evidently this finding taken together with the ability of Brn-3a to activate the HPV URR raises the possibility that elevated levels of Brn-3a may be critical for E6 and E7 gene expression in cervical cancer. We have therefore investigated the effect of artificially manipulating the levels of Brn-3a on HPV gene expression in cervical cancer cell lines and on their growth and transformation characteristics.

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The Brn-3a Transcription Factor Plays a Critical Role in Regulating Human Papilloma Virus Gene Expression and Determining the Growth Characteristics of Cervical Cancer Cells*

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The abbreviations used are: HPV, human papilloma virus; URR, upstream regulatory region; CIN, cervical intra-epithelial neoplasia.
MATERIALS AND METHODS

Plasmid Constructs—The expression vector pLTRpoly(ATCC) containing the full-length cDNAs of the class IV POU domain transcription factors Brn-3a and Brn-3b under the Moloney murine leukemia virus promoter have previously been described (27). The antisense Brn-3a construct was cloned within the pJ5 vector polylinker under the control of the

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**Fig. 1.** Panel a, Western blot showing Brn-3a and actin levels in SiHa cells either transfected with expression vector lacking any insert (track 1) or transfected with Brn-3a antisense vector and grown in the presence of dexamethasone (tracks 2 and 3) or in the absence of dexamethasone (tracks 4 and 5). Panels b and c, quantitative determination of the levels of Brn-3a protein based on densitometric scanning of data of the type shown in panel a in parental SiHa (cross, panel b) or C-33 cells (cross, panel c) cells or clonal cell lines transfected with expression vector lacking any insert (pci-neo, □), Brn-3a expression vector (●), Brn-3b expression vector (★), or a vector expressing the antisense strand of the Brn-3a gene (●) and then grown in the presence (■) or absence (●) of dexamethasone. Each data point shows a different independently isolated clonal cell line.
glucocorticoid-inducible mouse mammary tumor virus promoter (28).

Stable Transfection and Isolation of Clonal Cell Lines—SiHa (ATCC) and C33 (ATCC) cell lines were grown in minimum essential medium (Eagle’s) with Earle’s buffered saline solution supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate. The Brn-3 expression vectors were cotransfected with pci-neo (Promega) neomycin-resistant vector into both cell lines by calcium phosphate-mediated transfection method (30). Typically, 15 μg of the respective recombinants plus 3 μg of the neomycin-resistant plasmid were co-transfected into 80% subconfluent SiHa and C33 cells in 10-cm plates, and media were supplemented subsequently with G418 (Life Technologies, Inc.) dissolved in G418 supplemented media with or without dexamethasone to a final concentration of 0.33% was overlaid in triplicate 60-mm plates containing 0.5% low melting point agarose. Putative clonal or parental cells were isolated with cloning rings and cultured in medium supplemented with 800 μg/ml G418. Antisense and control clones were treated with 1 μM dexamethasone 24 h before protein extraction.

Western Blotting—Harvested cells were resuspended in 100 μl of extraction buffer (20 mM Hepes (pH 7.8), 450 mM NaCl, 0.4 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol 0.05 mM phenylmethylsulphonyl fluoride) and freeze-thawed. The protein concentration of the supernatant was determined, and the samples used for SDS-polyacrylamide gel electrophoresis as earlier reported (23) although with some modifications for HPV-E6 protein analysis with shorter SDS-polyacrylamide gel electrophoresis resolution time. The gel was blotted onto membrane (Amersham Pharmacia Biotech), and the membrane was blocked for 2 h with 10% Marvel (fat-free milk) and incubated with 1:500 HPV-16 E6 antibody (Santa Cruz) for 16 h overnight at 4 °C; washed 5 times with 0.1% Tween 20, then incubated with horseradish peroxidase-conjugated mouse secondary antibody (Santa Cruz) for 1 h.

RESULTS

To investigate the effect of manipulating the expression of Brn-3a in cervical cancer cell lines, we utilized the SiHa cell line, which contains a single integrated HPV-16 genome and, for comparison, the C33-transformed cervical cell line, which does not contain any HPV DNA (24–26). To overexpress Brn-3a, the cells were transfected with an expression vector in which expression of Brn-3a is driven by the moloney murine leukemia virus promoter, which we have previously used to successfully overexpress Brn-3a in neuronal cells (27). A similar vector was also used to overexpress Brn-3b in these cells for comparison. Similarly, to reduce the level of endogenous Brn-3a, we used a construct in which an antisense transcript of

FIG. 2. Levels of HPV E6 protein in the various cell lines. Panel a shows a typical Western blot with the antibodies to E6 (A) or actin control (B) by using extracts from SiHa neo cells grown in the presence (track 1) or absence (track 2) of dexamethasone, the Brn-3a antisense clone 3 grown in the presence (track 3) or the absence (track 4) of dexamethasone, and cells overexpressing Brn-3a (track 5) or Brn-3b (track 6) in the absence of dexamethasone. Panel b shows quantitative data for parental SiHa cells (SiHa) or clonal cell lines transfected with Brn-3a expression vector (SiHaA), Brn-3b expression vector (SiHaB), or two different cell lines transfected with the Brn-3a antisense vector (−3A clone 3 (−3A3) and −3A clone 5 (−3A5)). Data for the parental cells or the antisense cells are shown for cells grown in the absence or presence (dex) of dexamethasone.

FIG. 3. Cell growth curves of SiHa cells. The growth rate of parental SiHa cells is compared with that of SiHa cells transfected with empty expression vector (neo), Brn-3a expression vector (A), Brn-3b expression vector (B), or the antisense Brn-3a (−3A) vector in the presence or absence of dexamethasone. Similar results were obtained in three independent experiments. Panel a: ShHa; *, SiHaB. Panel b: ShHa; ●, ShHa + dexamethasone; ○, SiHaA.
Brn-3a is produced under the control of the glucocorticoid-inducible mouse mammary tumor virus promoter, which we have similarly previously used to reduce Brn-3a levels in neuronal cells (28). Similar transfections were also carried out using the expression vectors lacking any insert to produce control cell lines. In each case, stably transfected cell lines were selected on the basis of their neomycin resistance, which was encoded on the plasmid vector. In each case, several cell lines were isolated from different culture plates to control for clonal variation.

Clonal cell lines isolated in this way were first tested to determine whether they contained altered levels of Brn-3a as determined by Western blotting with a specific antibody. In these experiments (Fig. 1) clear overexpression of Brn-3a was observed in several cell lines transfected with the Brn-3a expression vector compared either to parental untransfected cells, cells transfected with the expression vector alone, or cells transfected with the Brn-3b expression vector. In contrast, several cell lines obtained by transfection with the Brn-3a antisense construct showed only minimal reduction of Brn-3a levels in the absence of dexamethasone to induce the antisense construct. However, a clear reduction in Brn-3a levels was observed in several of these cell lines when the cells were treated with dexamethasone, resulting in the induction of the antisense construct. This effect was observed in both the SiHa cells and in the C-33 cells transfected with the Brn-3a antisense construct (Fig. 1). In contrast, no effect of dexamethasone on endogenous Brn-3a levels was observed in either of the parental cell lines when treated with dexamethasone or in the cell lines transfected with expression vector lacking any insert (data not shown). Similarly, no alteration in exogenous Brn-3a levels in response to dexamethasone was observed in the cell lines obtained by transfection with the Brn-3a sense construct under the control of the Moloney murine leukemia virus promoter.

These data thus indicate that the cell lines engineered to overexpress Brn-3a do indeed show a specific elevation of Brn-3a levels, whereas the antisense cell lines show decreased expression of Brn-3a, particularly when grown in the presence of dexamethasone to induce the antisense construct. The cell lines showing the greatest elevations or reductions in Brn-3a levels were selected for further study. Similarly, the SiHa and C33 cell lines over-engineered to express Brn-3b showed a specific elevation of Brn-3b levels, which was not observed in the other cell lines (data not shown).

To determine whether these alterations in Brn-3a and Brn-3b levels did produce a change in the level of HPV gene expression driven by the URR promoter, the cellular extracts were also Western-blotted with antibody to the HPV E6 protein. In these experiments (Fig. 2) the SiHa cells engineered to overexpress Brn-3a showed no significant increase in HPV expression over the control parental SiHa cells or SiHa cells containing only plasmid vector, suggesting that HPV gene expression is likely to be maximally stimulated by the endogenous Brn-3a present in the SiHa cells. Interestingly, however, the cells engineered to overexpress Brn-3b showed a small decrease in HPV gene expression. A small decrease in HPV...
gene expression was also observed in both the cell lines containing the antisense construct. Most importantly, a significant further decrease in HPV gene expression was observed in both these cell lines upon treatment with dexamethasone to fully induce the antisense constructs. In contrast, no effect of dexamethasone on HPV expression was observed in the parental SiHa cells (Fig. 2), confirming that this effect was dependent upon the activation of the antisense construct by dexamethasone rather than on a direct inhibitory effect of dexamethasone on HPV gene expression. Interestingly, the decrease in HPV gene expression was greatest in antisense cell line 5, paralleling the greater reduction in Brn-3a levels in this cell line compared with the antisense cell line 3. As expected, no HPV gene expression was detected in any of the cell lines derived from C-33 cells, which are not transformed with HPV.

These data indicate therefore that the HPV gene expression that occurs in the SiHa cell line appears to be dependent upon the expression of Brn-3a in these cells since it can be specifically reduced by decreasing Brn-3a levels using an antisense approach. We therefore wished to establish whether such alteration in HPV gene expression mediated via Brn-3a resulted in alterations in the growth rate of the manipulated cells. Evidently, the C33 cells serve as an important control for these experiments, since any direct effect of manipulating Brn-3a levels on cellular growth would also be observed in these cells, whereas this would not be the case if the effect in SiHa cells is mediated via the alteration in HPV gene expression that would not occur in the C33 cells. We therefore measured the growth rate of the various different clones over a 72-h period. In the experiments with the SiHa clones, the parental SiHa cells and the cells transfected with empty expression vector alone showed a similar growth rate (Fig. 3a), indicating that the selection of stably transfected cell lines does not produce cell lines with enhanced growth rates. Interestingly, overexpression of Brn-3a resulted in a marginally enhanced growth rate of the SiHa cells, whereas overexpression of Brn-3b produced a significantly reduced growth rate (Fig. 3a). Most importantly, although the cells engineered with antisense Brn-3a showed a similar growth rate to parental cells in the absence of dexamethasone, their growth rate was dramatically reduced by treatment by dexamethasone, although this treatment had no effect on the growth of parental cells (Fig. 3b).

These experiments thus indicate that the reduced Brn-3a expression in the antisense SiHa cells is paralleled not only by reduced HPV gene expression but also by reduced growth rate. In similar experiments in the HPV-negative C-33 cells (Fig. 4), all the cell lines showed similar growth rates. Hence, manipulating the expression of Brn-3a or Brn-3b in a cervical cell line that does not express HPV does not result in altered growth rates.

As well as measuring the effect of manipulating Brn-3a expression on cellular growth rate, we also wished to determine whether such manipulation would affect the saturation density of the cells, since the loss of contact inhibition resulting in growth to higher densities is characteristic of cancer cells. The various cell lines were therefore plated out and grown over a period of several days to determine their saturation density. In these experiments (Fig. 5), similar saturation densities were observed in the parental SiHa cells, the cells transfected with plasmid expression vector alone, and the cells overexpressing Brn-3a. However, a clearly reduced saturation density was observed in the SiHa cells overexpressing Brn-3b (Fig. 5a). Similarly, two distinct SiHa cell lines containing the transfected antisense construct showed a clear reduction in saturation density compared with the parental cells (Fig. 5b). This reduction was greater in cell line 5 compared with cell line 3, paralleling the greater reduction in HPV gene expression in this cell line (see Fig. 2). Moreover, the saturation density of both the antisense cell lines was further reduced by full induction of antisense expression using dexamethasone, whereas no effect on saturation density was observed when the parental cells were treated in this way, confirming that this effect was specific to the cells containing the antisense construct (Fig. 5b). As in the cell growth experiments, all the C-33-derived cell clones showed similar saturation densities that were unaffected by dexamethasone (Fig. 6), indicating that the effects in SiHa cells correlate with the effect of Brn-3a on HPV gene expression.

Having established the effect of manipulating Brn-3a expression on the growth and saturation density of the cell lines when grown attached to culture dishes, we wished to determine the effect of such manipulation on their ability to grow in an anchorage-independent manner, since this is an important feature of tumor cells necessary for their growth in vivo. We therefore measured the ability of the various cell lines to form colonies in soft agar. As indicated in Fig. 7a, the SiHa cells showed a clear ability to form colonies in soft agar as expected, and this was not affected in the cells containing the plasmid expression vector or in the cells overexpressing Brn-3a.

However, a significantly reduced rate of colony formation in soft agar was observed in the cells overexpressing Brn-3b, paralleling their reduced growth rate and saturation density.
when grown attached to culture dishes (p < 0.05 for SiHa-neo compared with SiHa B). Moreover, a still greater reduction in colony formation of approximately 4-fold was observed in the two different cell lines containing the antisense Brn-3a construct (Fig. 7a). This colony formation was reduced even further upon treatment of the antisense cells with dexamethasone, with colony formation being virtually undetectable in cell line 5, paralleling its greater reduction in HPV gene expression (p < 0.05 for each antisense line compared with parental cells). This effect of dexamethasone was specific to the antisense cell lines, since no effect of dexamethasone on colony formation was observed in the parental cells. To determine whether these effects of manipulating Brn-3a levels on anchorage-independent growth were dependent upon the altered level of HPV gene expression, we carried out similar experiments in the C-33-derived cell lines. As illustrated in Fig. 7b, however, all the various cell lines showed a similar ability to form colonies in soft agar, which was not in any way affected by the alteration of Brn-3a or Brn-3b levels, with no significant differences being noted between the various cell lines (p > 0.05 in all cases). Hence, the effects on anchorage-independent growth observed in the SiHa cell lines are correlated with the effect of Brn-3a on HPV gene expression in the same manner as the effect on the growth of cells attached to culture dishes.

**DISCUSSION**

In this report we have demonstrated for the first time that the manipulation of Brn-3a expression can affect the levels of gene expression from an integrated HPV genome in a transformed cervical cell line. Thus, SiHa cell lines transfected with an antisense Brn-3a expression plasmid showed a clearly reduced expression of HPV. Hence, the expression of the single endogenous HPV genome in SiHa cells appears to depend upon the expression of Brn-3a in these cells such that when Brn-3a expression is reduced, HPV gene expression is correspondingly reduced. This effect evidently parallels our previous finding that the ATGCAATT motif in the HPV URR can be transactivated by Brn-3a in co-transfection assays involving promoter-reporter constructs (22) and extends such transactivation to an endogenous HPV genome. Interestingly, despite the increased Brn-3a levels observed in cells transfected with an expression vector for Brn-3a, the levels of HPV gene expression were not significantly increased, suggesting that HPV gene expression is already maximally stimulated in SiHa cells by the significant level of endogenous Brn-3a in these cells. Interestingly however, HPV gene expression could be reduced by overexpressing Brn-3b in SiHa cells, paralleling the ability of Brn-3b to repress URR activity via the ATGCAATT motif in co-transfection experiments (22). These experiments thus establish Brn-3a expression as being critical for the maintenance of HPV gene transcription in a cervical cancer cell line. In this report we have also demon-
strated that the growth characteristics of such a cell line are similarly dependent upon Brn-3a. Thus, the inhibition of Brn-3a expression using an antisense approach led to reduced cellular growth rate, saturation density, and the ability to grow in an anchorage-independent manner. Several lines of evidence indicate that this effect is dependent upon the ability of Brn-3a to modulate HPV gene expression rather than to a direct effect of Brn-3a on the cell. Thus, no effect of reduced Brn-3a levels on cellular growth, saturation density, or anchorage independence was observed in the C-33 cells, which showed a similar reduction in Brn-3a levels but that are not transformed with HPV. Similarly, overexpression of Brn-3b in the SiHa cells, which also reduced HPV gene expression, also resulted in reduced growth rate, saturation density, and anchorage-independent growth, although the effects were not as dramatic as reducing Brn-3a levels, paralleling the less dramatic effect of overexpressing Brn-3b on HPV gene expression. Last, it should be noted that in the C4–1 cervical carcinoma cell line, reduction of Brn-3a levels, although the effects were not as dramatic as reducing Brn-3a levels, also reduced HPV gene expression, also resulted in reduced cellular proliferation (29).

Thus, simply by manipulating Brn-3a levels it is possible to alter HPV oncogene expression and thereby alter the growth characteristics of the tumor cells in terms not only of growth rate and independence from contact inhibition but, most importantly, in terms of anchorage independence, which is a key requirement for tumorigenesis in vivo. This association of Brn-3a with HPV gene expression and the characteristics of transformed cervical cells is of particular interest in view of our previous findings that Brn-3a is overexpressed in the transformation zone of women with CIN3 compared with women with no detectable cervical abnormality (23). Such overexpression of Brn-3a is likely therefore, in view of our current results, to play a key role in the elevated HPV gene expression observed in the transformed cells, which is critical for oncogenic transformation.

Hence the elevated levels of Brn-3a observed in CIN3 material and in cervical cancer cell lines appear to play a key role in the elevated expression of HPV and thereby in determining the transformed phenotype. These considerations evidently focus attention on the manner in which Brn-3a expression becomes elevated in women with CIN3. In our previous study (23) we were able to show that similar elevated expression of Brn-3a occurs in histologically normal segments of the cervix adjacent to the CIN3 region that do not contain detectable HPV DNA or RNA, and we have now extended these findings to show that Brn-3a expression is elevated throughout the cervix in women with CIN3.2 This widespread elevation in Brn-3a levels in women with CIN3 may be dependent upon their exposure to an environmental factor that raises Brn-3a levels, or alternatively, could reflect a genetic difference in the Brn-3a gene regulatory region, which results in elevated expression of Brn-3a in these women. In this latter case, this genetic polymorphism would represent a risk factor for cervical cancer similar to having a p53 gene encoding a protein with an arginine at position 72, resulting in enhanced degradation by the HPV E6 protein (6).

Whatever the cause of the elevated level of Brn-3a, however, it is clear that in individuals having such elevation, infection with HPV-16 or HPV-18 will result in the activation of the HPV URR, leading to E6 and E7 expression and cellular alterations in the cervical transformation zone at the junction of the endocervix and the ectocervix, where cervical tumors appear. Hence the elevated levels of Brn-3a, whether caused by environmental or genetic causes, would play a critical role in activation of viral transcription and disease progression, although other factors such as viral type, viral distribution, and cellular susceptibility to transformation would be responsible for the precise localization of the malignant lesions.

Most importantly, the fact that the level of HPV gene expression and the abnormal growth characteristics of cervical cancer cells can be reversed by reduction of Brn-3a expression makes this factor an attractive target for therapeutic intervention. This could involve the reduction of endogenous Brn-3a expression either by pharmacological manipulations to reduce the activity of the Brn-3a gene promoter or by the use of gene delivery vectors to deliver Brn-3a antisense constructs similar to those utilized here. To investigate further the potential utility of such forms of therapy, it will be necessary to investigate whether the SiHa cells with reduced Brn-3a levels show reduced tumorigenicity when grown in nude mice and also whether the growth rate of an established tumor can be manipulated in vivo by using viral vectors expressing the antisense strand of the Brn-3a gene. It is already clear, however, from the experiments described here that the Brn-3a factor, which is overexpressed in women with CIN3, plays a key role in HPV gene transcription and thereby regulates the growth characteristics of cervical carcinoma cells.

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