Nuclear Localization of the Testis Determining Gene Product SRY

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Abstract. We have studied the expression of the human SRY protein (termed p27SRY) in two different cell lines by using specific antibodies. Confocal microscopy enabled us to localize p27SRY precisely in the nucleus in a discrete punctuate pattern. Furthermore, through microinjection experiments, we have demonstrated that the localization of the p27SRY protein into the nucleus was an event involving the NH2-terminal part of the high mobility group (HMG) domain. With the help of several synthetic peptides and various p27SRY mutants, we have characterized a bipartite basic motif in this part of the protein corresponding to a nuclear localization signal. This nuclear localization signal appears to be highly conserved in SRY box- and HMB box-containing proteins, suggesting common properties of nuclear targeting within the HMG box protein family.

In mammals, the presence or absence of a Y chromosome is known to correlate with male or female phenotype. In the absence of the Y chromosome, undifferentiated gonads (fetal genital ridges) develop as ovaries, while in its presence they will develop as testes. It is therefore believed that a genetic switch named testis determining factor (TDF) and localized on the Y chromosome diverts the primordial gonads from the ovarian pathway towards male differentiation to form testes. In human, this event takes place at about six weeks of embryonic life. Given that Sertoli cells are the first testicular cell type to differentiate (Jost et al., 1981), the genetic switch is believed to commit a specific cell lineage to the Sertoli cell differentiation pathway, which is then responsible for the production of the anti-Müllerian hormone (AMH also known as Müllerian inhibiting substance or MIS) (Josso et al., 1993).

Using sex-reversed patients, a single Y-located gene, termed sex determining region Y chromosome (SRY) in human and Sry in mice, has recently been identified and cloned (Sinclair et al., 1990; Gubbay et al., 1990). Growing evidence supports SRY identity with TDF: first, de novo mutations affecting the human SRY open reading frame are present in some 46, XY females with complete gonadal dysgenesis (Berta et al., 1990; Jäger et al., 1990). Next, the murine homologue Sry was shown to be expressed in the normal developing embryo in the genital ridges from 10.5-12.5-d postcoitum just prior to the differentiation of the genital ridges into testes (Koopman et al., 1990). Finally, addition of a 14-kb DNA fragment derived from mouse Y chromosome and including the Sry gene, induced transgenic mice with a XX chromosomal background to develop as phenotypic males (Koopman et al., 1991). However, the mode of action of the SRY gene product remains puzzling. A predominant feature of the SRY protein is a 79-amino acid motif referred to as the high mobility group (HMG) box, a conserved domain in many DNA-binding proteins including several transcription factors (Ner, 1992). This led to the proposition that SRY could be a DNA-binding protein. Thus, by analogy it could play the role of a transcriptional regulator of downstream genes involved in the sex-determining pathway. Preliminary results confirmed this assumption: using either the SRY open reading frame or the potential DNA-binding motif in gel shift experiments, the ability of SRY protein to bind DNA in a sequence-specific manner was demonstrated (Harley et al., 1991; Nasrin et al., 1991; Denny et al., 1992). The specificity of the binding was assessed by the demonstration that sex-reversed females with point mutations in the SRY gene, as described in approximately 15% of XY females (Berta et al., 1990; Hawkins et al., 1992), lose part or all of this binding activity (Harley et al., 1992; Poulat et al., 1994). Furthermore, like other members of the high mobility group family of DNA-binding proteins, SRY has been shown to interact with DNA double helix in the minor groove (Van de Watering and Clevers, 1992) inducing a substantial bend in target DNA (Giese et al., 1992; Ferrari et al., 1992). Finally, SRY protein binds with high affinity to cruciform
in a non sequence specific manner (Ferrari et al., 1992).

In this study, we describe the production and characterization of an antibody directed against the human testis determining SRY gene product (also termed p27SRY). Using this specific antibody, we provide information about the nuclear localization of the p27SRY protein in human cells expressing the endogenous SRY gene. The nuclear localization signal of the p27SRY protein has been defined following microinjection of several SRY-derived peptides and p27SRY mutant expression plasmids.

**Materials and Methods**

**Construction of p27SRY Protein Expression Plasmid, Production of p27SRY Protein**

For p27SRY expression in mammalian cells, DNA encoding the SRY open reading frame (Sinclair et al., 1990) was subcloned into the pBluescript expression vector (in which the DNA sequence of interest is under control of a SV-40 promoter) (Morgensen and Land, 1990). A HindIII fragment isolated from the SRY containing clone pY53.3 (Sinclair et al., 1990) was ligated into the pBluescript vector previously cut with Sma I (GIBCO-BRL, Erangy, France). This construction was termed p3SRY. Mutant forms of SRY gene were produced using the Transformer Mutagenesis Kit (Clontech, Ozyme, France).

For p27SRY expression in bacteria, DNA encoding the SRY open reading frame was PCR amplified and subcloned in the pOTSV bacterial expression vector system (Schatzman et al., 1987) at the BamHI/Xhol sites. The resulting plasmid was used to transform the AR58 bacterial strain as previously described (Schatzman et al., 1987). Cells were grown at 30°C and induced at 42°C for 90 min. After purification, inclusion bodies were solubilized in Laemmli buffer (40 mM Tris-HCl, pH 6.8, 1% SDS, 50 mM DTT, 7.5% glycerol).

**Peptide Synthesis**

Peptides were produced using continuous flow solid-phase synthesis essentially as described previously (Poulat et al., 1992), and purified by semi-preparative RP-HPLC. Sequences were then shown to be correct by amino acid analysis and the purity was further checked by HPLC and HPCE.

SRY80 peptide corresponds to the entire SRY box motif (Sinclair et al., 1990) and SRY64 to the same sequence lacking the 16 NH2-terminal residues. SKY21 refers to the potential nuclear localization sequence from the SRY DNA binding motif, in sequence DRVKRPMAFIWSDQRKRC (corresponding to amino acids 58 to 78 of human p27SRY). SKY17 corresponds to amino acids 40 to 56 (CNSKYQCETGENSKGNV) of the p27SRY protein NH2-terminal region. Peptide SV40 (KKKRKVC) corresponds to the natural LNS of the large T antigen from the SV-40 simian virus. All peptides were coupled to rabbit IgG (Sigma, France) using m-maleidobenzoyl-N-hydroxysulfosuccinimide ester (Pierce, Interchim, France). Peptide-protein conjugates were filtered on PD10 column (Pharmacia, France) equilibrated in PBS.

**Antibody Production and Purification**

Synthetic peptide (SKY17) was coupled to thyroglobulin (Sigma, France) or to BSA (Sigma) as described before. Male New Zealand rabbits were injected with 300 μg of thyroglobulin peptide conjugate mixed with complete Freund’s adjuvant (Sigma). Rabbits were injected every two weeks with 100 μg of the same protein preparation in incomplete Freund’s adjuvant (Sigma), and bled 10 days after each injection. Polyclonal antisera were purified using a BSA–peptide conjugate coupled to CNBr Sepharose (Pharmacia, France) as previously described (Girard et al., 1991) and will be further referred to as Y127.3 antibody.

**Western Blotting**

Nuclei were prepared as follows: cells were washed with cold PBS, collected with a rubber policeman and lysed with a Dounce homogenizer in 0.5% NP-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2 buffer. Nuclei were purified by centrifugation (3000 g, 8 min, 4°C) on a sucrose cushion (30% sucrose, 50 mM Tris-HCl, pH 8.3, 5 mM MgCl2, 0.1 mM EDTA), and then lysed in Laemmli buffer, and sonicated for 20 s. Inclusion bodies containing the p27SRY recombinant protein were solubilized in the same buffer. All samples were boiled for 5 min, separated on 12.5% SDS-PAGE and transferred to nitrocellulose. Nitrocellulose sheets were saturated in 8% nonfat-dried milk in 25 mM Tris HCl, pH 7.5, 200 mM NaCl (TBS) for 1 h, and then incubated with anti-SRY antibody (conc. 0.3 mg/ml) diluted 1:100 in TBS for 1 h at room temperature. After extensive washing in TBS containing 0.05% Tween 20 and 1 h resaturation in 8% milk, horseradish peroxidase conjugated to anti-rabbit antibodies (Amersham) was added at 1:10000 for 1 h. Detection of the immune signal was carried out by using the ECL detection system (Amersham). For competition experiments, 7 μg of Y127.3 were allowed to react with peptides SKY17 or SKY21 in a final volume of 200 μl for 1 h at room temperature, and then diluted to 1:100 in TBS and used for Western blotting as described above.

**Cell Culture**

NT2D1 cells (N-Tera 2, clone DI, a human pluripotent embryonic carcinoma cell line, ATCC No. CRL 1973) were obtained from the American Type Culture Collection (ATCC, Biovaley, France). H568 cells (a human adult fibroblastic cell line, ECACC No. 89051701) and HeLa cells (a human cervix carcinoma cell line, ECACC No. 85060701) were obtained from the European collection of animal cell culture (ECACC, Sophia-antipolis, France). NT2D1 cells, HeLa cells and human H568 or H8 fibroblasts were cultured in DME (Imperial Laboratories, Flobio, France) containing 10% fetal calf serum (FCS) (GIBCO-BRL), penicillin/streptomycin, and 2 mM glutamine as described (Girard et al., 1992). For microinjection experiments, cells were plated onto acid washed glass coverslips essentially as described previously (Fernandez et al., 1991) and used 2-3 d after subculture. Human embryonic fibroblasts H8 were obtained from a 2 mm 3 skin fragment of a 4-wk human male embryo obtained after voluntary abortion. Cells were serially passaged every 3 d essentially as described by Todaro and Green (1963). After 33 passages, cells were subjected to limited dilution and individual clones 10-23 grown up for use.

**Establishment of a p27SRY Expressing HeLa Cell Line**

To obtain a reference cell line, we established a p27SRY protein expressing stable transfectant. Since HeLa cells cannot express the SRY gene product because they do not contain a Y chromosome, we cotransfected this cell line with p3SRY vector and pDNA Neo (Clontech, Ozyme, France) to establish a stable transfectant for the human SRY gene. Briefly, 5 X 105 HeLa cells were transfected with both plasmids for one night at 37°C using Lipofectin reagent (GIBCO-BRL). Cells were then washed, diluted, and incubated for 2 wk in the presence of 500 μg Neomycin (GIBCO-BRL) per ml of 10% FCS DME. Cell clones were then collected, amplified, and analyzed for SRY gene expression by RT-PCR detection transcription followed by polymerase chain reaction (RT-PCR) experiments. Positive and negative clones were then analyzed for p27SRY protein expression using immunoblotting experiments as previously described. A p27SRY expressing clone was next selected and termed “HeLa SRYB3.”

**Embryonic Tissue Preparation**

8-wk-old male or female fetuses were obtained from therapeutic termination (weeks of gestation). Sections of unfixed frozen tissues (10-μm thick) were cut on a cryostat at −25°C. Sections were placed onto coated polylysine slides, dried for 1 h at room temperature, and then wrapped in cling film and stored at −20°C. Prior to immunolocalization, wrapped sections were fixed with −20°C methanol for 5 min, and rehydrated in PBS. Sections were probed using the Y127.3 antibody and then processed as described below. DNA visualization was obtained using Hoechst 33236 staining.

**Microinjection and Immunofluorescence**

Cells growing on glass coverslips were microinjected with plasmid DNA essentially as follows. After allowing the cells to grow, selected areas were demarked on the coverslips by removing surrounding cells with fine forceps. After transferring the coverslips to prewarmed (37°C) PBS (to prevent the salts in DME from precipitating DNA), the nuclei of cells were injected by introducing the needle into the center of the nuclei, withdrawing it slightly to prevent nuclear material from blocking the needle point, and applying pressure. 10-50 cells were routinely injected per experiment. For
after preincubation of the Y127.3 antibody with either the 40 promoter (HeLa SRYB3) (Fig. 1, lane 5). In all cases, the preimmune serum, and could be specifically abolished this 27-kD protein immunoreactivity was not present with the 27 kD protein in nuclear extracts derived from HeLa cells transduced to express recombinant p27SRY protein (Fig. 1, lane 4). However, Y127.3 did recognize a major 27-kD protein in NT2D1 cell nuclear extract (lane 8). H8 cell (passage 10) nuclear extract (termed H8.10). White arrows show the position at which p27SRY migrates. Proteins were run on a 12.5% polyacrylamide gel. The migration of the mol wt standards, trypsin inhibitor (20 kD), carbonic anhydrase (31 kD), ovalbumin (43 kD) and albumin (67 kD) have been marked on the nitrocellulose filter after Ponceau red staining and before immunostaining. Molecular weights are given in kD. The estimated molecular weight of SRY protein is around 27 kD.

Figure 1. Western blotting characterization of the human p27SRY protein. Monospecific oligoclonal antibodies (Y127.3) raised against a part of the amino terminal domain of p27SRY were used as a probe for p27SRY. Shown are (lane 1) bacteria with empty plasmid expression vector, (lane 2) bacteria induced to express p27SRY protein, (lane 3) HS68 cell nuclear extract, (lane 4) Hela cell nuclear extract, (lane 5) SRY stably transfected Hela cell nuclear extract (Hela SRYB3), (lane 6) NT2D1 cell nuclear extract, (lane 7) H8 cell (passage 2) nuclear extract (termed H8.2), and (lane 8) H8 cell (passage 10) nuclear extract (termed H8.10). White arrows show the position at which p27SRY migrates. Proteins were run on a 12.5% polyacrylamide gel. The migration of the mol wt standards, trypsin inhibitor (20 kD), carbonic anhydrase (31 kD), ovalbumin (43 kD) and albumin (67 kD) have been marked on the nitrocellulose filter after Ponceau red staining and before immunostaining. Molecular weights are given in kD. The estimated molecular weight of SRY protein is around 27 kD.

Results

Production of a Specific Antibody against the SRY Protein and Characterization of Two SRY Expressing Cell Lines

To examine the expression and cellular distribution of the human SRY gene product in mammalian cells, an antibody against a peptide derived from the sequence of the p27SRY protein was raised. This sequence termed SRY17 corresponds to amino acids 40 to 56 of the human p27SRY protein (Sinclair et al., 1990). This sequence is excluded from the putative DNA binding domain of p27SRY and was chosen so as to minimize potential cross-reactivity with related proteins, the so-called SOX proteins ("SRY bOX") (Griffiths, 1991) which display an homologous HMG domain with the p27SRY protein.

The specificity of the resulting affinity purified antibody Y127.3 was initially determined using Western blotting experiments. As shown in Fig. 1, Y127.3 detects a major protein at around 27 kD in total cell lysates from bacteria induced to express recombinant p27SRY protein (Fig. 1, lane 2). This molecular weight is in good agreement with the calculated molecular weight deduced from the open reading frame of the SRY gene. By contrast, no signal was present in bacterial lysates lacking the p27SRY encoding plasmid (Fig. 1, lane 1) or from the nuclear extracts of two different cell lines: HS68, a human male adult fibroblast cell line (Fig. 1, lane 3) and HeLa, a human cervix carcinoma cell line (Fig. 1, lane 4). However, Y127.3 did recognize a major 27-kD protein in nuclear extracts derived from HeLa cells transfected with human SRY gene under the regulation of the SV40 promoter (HeLa SRYB3) (Fig. 1, lane 5). In all cases, this 27-kD protein immunoreactivity was not present with the preimmune serum, and could be specifically abolished after preincubation of the Y127.3 antibody with either the immunizing peptide (SRY17) or purified p27SRY protein (data not shown); in contrast, an irrelevant peptide (SRY21), was unable to abolish the Y127.3 immunoreactivity (data not shown).

Endogenous expression of the p27SRY protein was screened in two different cell lines: N-Tera 2 clone D1 (NT2D1), a pluripotent embryonic carcinoma cell line (An-drews et al., 1984), and H8, a human embryonic non transformed cell line developed in our laboratory. Using the Y127.3 antibody in Western blotting experiments, a protein of 27 kD was detected in NT2D1 nuclear extracts, in good agreement with the molecular weight of p27SRY (Fig. 1, lane 6). However, this immunoreactivity was only detectable in H8 nuclear extracts of cells from passage 1 to 9 (Fig. 1, lane 7). Expression of the Y127.3 immunoreactive protein decreased rapidly with cellular passages, and completely disappeared after ten cell passages (Fig. 1, lane 8). Interestingly the protein detected in the NT2D1 and H8 cells migrated with the same molecular weight as the protein expressed in the Hela B3 cells, but in all three cases it was different when compared to the bacterially expressed protein. This difference of electrophoretic mobility between bacterially or cellurally expressed proteins could result from post-translational modifications of p27SRY in eukaryotic cells, since the human SRY transcript is encoded by a monoeconomic gene (Su et al., 1993; Behlke et al., 1993). Furthermore, we have observed phosphorylation of p27SRY protein.
in vitro by a number of purified kinases and that might account for these differences (Poulat, F., and P. Berta, manuscript in preparation).

Tricoli and co-workers did not detect any SRY gene expression in the original cell line NTERa2, using RT-PCR experiments (Tricoli et al., 1993). However the subclone examined here (clone D1) has been recently described to be positive for the SRY transcript (Clepet et al., 1993).

Western blotting experiments using total cellular extracts were also performed. In this case, identification of the p27SRY protein was very difficult to obtain reproducibly, probably reflecting the low overall quantity of p27SRY protein present in these cells. Furthermore, when Western blotting experiments were carried out using the cytoplasmic fraction of the cellular extract, no significant immunoreactivity with the antibody Y127.3 was detected (data not shown), suggesting that the p27SRY protein is essentially nuclear.

From these data, we can conclude that the 27-kD protein detected with the Y127.3 antibody in cellular extracts is indeed the p27SRY protein encoded by the human SRY gene.

**Nuclear Localization of p27SRY Protein in NT2DI, H8, and Embryonal Cells**

To investigate the cellular localization of the p27SRY protein, we examined a number of cell lines for the distribution of p27SRY using indirect immunofluorescence with the help of the Y127.3 antibody characterized above. Cells were then examined by confocal scanning laser microscopy, with the confocal plane passing through the center of the cells. As shown in Fig. 2, p27SRY staining appears essentially localized in the nucleus of the NT2DI cells throughout all stages of interphase (Fig. 2, D and E). The low level of cytoplasmic staining which can be detected is similar in intensity to the background cytoplasmic staining observed with preimmune

**Figure 2.** Two cell lines spontaneously expressing p27SRY protein show a discrete nuclear punctuated immunofluorescent staining. Asynchronously growing cells H8 and NT2DI were fixed and processed for immunofluorescence using Y127.3 antibody. Using confocal microscopy, H8 cells from passage 2 (A) and NT2DI cells (D) display a nuclear staining made up of bright spots of immunofluorescence. When several cells are examined in the same field, the immunofluorescent staining is heterogeneous in intensity between the cells. Some cells show a very low level of p27SRY staining (white arrows, B: H8.2 cells culture and E: NT2DI cell culture), by contrast with bright staining visible in other cells in the field. When the antibody Y127.3 was preincubated with the immune peptide (SRY17') prior to the immunofluorescence experiments, no staining was detectable in each cell line (C and F). Bar, 5 μm.
serum (data not shown). The nuclear NT2D1 staining displays a brightly punctated pattern of immunofluorescent foci throughout the nucleus and could represent some important localized concentrations of p27SRY protein associated with chromatin.

Similar staining was also observed in human H8 embryonic fibroblasts across passages 1 to 9 (Fig. 2, A and B). This p27SRY staining completely disappeared after 10 passages (data not shown), confirming the previous Western blotting experiments.

In these two cell lines, the level of immunoreactivity with Y127.3 is not homogeneous as proved by the staining of different cells in the same micrographic field (Fig. 2, B and E): some cells display a strong Y127.3 staining, whereas others show very low if any immunoreactivity (Fig. 2, B and E, white arrows). Although the reasons for this are presently unclear, it could result of the use of asynchronously growing cell culture. This observation could not be explained by a heterogeneity of the cellular population since both NT2D1 and H8 cell lines were derived from a single clone. Moreover, NT2D1 cells were cultured at high density to prevent heterogeneous differentiation as recommended by the purchaser.

High resolution optical sectioning revealed that the p27SRY staining was always excluded from the nucleoli in the two cell lines (Fig. 3, A and C), as confirmed by phase contrast micrography (Fig. 3, B and D).

To confirm this nuclear localization, a preincubation of the affinity purified antibody with peptide SRY17 was carried out. As shown in Fig. 2 (C and F), this preincubation completely abolished the nuclear staining in both cell lines, strongly suggesting that the nuclear staining within the nucleus of NT2D1 and H8 cells represents the true localization of p27SRY protein in these two cell lines. Secondly, the absence of p27SRY expression in H8 cells after the tenth passage, as detected using Western blotting experiments was confirmed by the absence of immunofluorescence staining in the same cells (data not shown).

Finally, the immunolocalization of the SRY protein and its punctated pattern as observed in these two cell lines were confirmed using gonadal tissue from four 8-wk-old male fetuses (Fig. 4). Furthermore, this staining was also shown to be absent in a female embryo (Fig. 4, E). However, although the protein was always punctated and nuclear, we did observe differential levels of staining in different areas of the section. Since the present analysis included less than 50 sec-

![Figure 3. p27SRY is excluded from the nucleoli. Nuclei from H8.2 and NT2D1 cells were observed using confocal microscope. (A and C) H8.2 and NT2D1 cell nuclei where the p27SRY staining is excluded from the nucleoli (grey arrows). (B and D) Phase contrast micrographs of the H8.2 and NT2D1 cells nuclei showing the nucleoli (white arrows). Bar, 5 μm.](image-url)
The p27SRY protein expressed in the gonadal tissue of a male fetus is localized within the nucleus with a punctate immunostaining pattern. 10-μm-thick sections from male and female embryos were immunostained with Y127.3 anti-SRY antibody and Hoechst 33286 to visualize DNA, as described in Materials and Methods. Only the gonadal region is shown in each panel. (A and B) Immunofluorescence staining with Y127.3 revealed the nuclear localization of SRY gene product in only a subset of cells in the gonad. Shown in B is the DNA staining of the same cells. (C and D) Higher magnification reveal the punctate immunolocalization of the SRY gene product in the nuclei. (E and F) Absence of SRY staining (E) in the gonad of a female embryo. Shown in F is the corresponding Hoechst staining. Bars, 10 μm.

Localization of the p27SRY Protein in Microinjected Cells

To examine in more detail the mechanisms involved in the nuclear localization of p27SRY protein, we used microinjection experiments in human adult fibroblasts (HS68), a lineage negative for p27SRY expression.
Figure 5. Overexpression of both wild type human p27SRY protein and mutant p27SRY and mutYC in human fibroblast cell line reveals a constitutive nuclear localization. Plasmids encoding the full length SRY ORF (pJ3SRY) or a mutant unable to bind DNA (pJ3SRY mutYC) were injected into HS68 cells. After 12-15 h at 37°C, cells were fixed and localization of the p27SRY protein determined by indirect immunofluorescence with the anti-SRY antibody Y127.3. On immunofluorescence micrographs are: (A) the localization of wild type p27SRY protein; (B) an inert marker antibody revealing the injected cells with the pJ3SRY construct; (C) phase contrast micrograph in order to appreciate the background of the antibody in non-injected cells; (D) the localization of mutant p27SRY mutYC protein, and (E) a marker antibody co-injected with the plasmic pJ3SRY mutYC. Bar, 5 μm.

Experiments were carried out as follows. Asynchronously growing cells were microinjected into the nucleus with plasmids encoding the full length p27SRY protein under regulation of a strong eukaryotic promoter (SV-40). Microinjection solutions also included an inert mouse antibody in order to subsequently visualize injected cells. Cells were then allowed to grow at 37°C, 12 to 15 h, before processing for immunofluorescence with Y127.3 antibody. Results are presented in Fig. 5. Microinjected cells, identified with the staining of a coinjected marker antibody (Fig. 5 B), show a bright homogeneous nuclear staining for p27SRY in human fibroblasts (Fig. 5 A). Such nuclear staining was not present in the surrounding non-injected cells (identified by phase contrast micrography; Fig. 5 C). Cells microinjected with an antisense p27SRY coding sequence containing plasmid or with the empty plasmid alone had a staining pattern similar to the background (data not shown).

We conclude that recombinant p27SRY protein contains all the intrinsic properties to be translocated to the nucleus when microinjected in cells that normally do not express it.

Nuclear Retention of p27SRY Protein Does Not Depend on Its DNA Binding Activity

To investigate if the nuclear retention of p27SRY protein depends on its DNA binding capacity, a mutant form of this protein, unable to bind DNA (and based on the SRY gene sequence of a mutated XY female), was produced (Poulat et al., 1993). In this case, a tyrosine residue was changed for a cysteine at the position 127 of the p27SRY protein, a residue located in the COOH-terminal part of the HMG domain. Using gel shift assays, we have previously demonstrated that a complete loss of the DNA binding activity of the SRY gene product results from this mutation, at least in vitro. This mutation was reproduced in a pJ3SRY expression vector (termed pJ3SRY-mutYC). 12-15 h after microinjection of the expression vector in HS68 cells and subsequent staining with Y127.3 antibody, the p27SRY-mutYC is clearly nuclear (Fig. 5 D, p27SRY-mutYC protein; panel E, marker antibody). If nuclear retention was a function of a DNA binding activity, some cytoplasmic staining should be detected; however, even in the cells that strongly expressed p27SRY-mutYC, only nuclear staining could be detected. From these results, we can conclude that for p27SRY, retention in the nucleus and DNA binding activity of the protein are two independent functions.

A Nuclear Localization Signal Is Present in p27SRY HMG Box

To identify the nuclear localization signal (NLS) of the p27SRY protein more precisely, the intracellular distribution
Figure 6. Characteristics of the SRY-derived peptides. The origins of the various peptides derived from the SRY open reading frame used in these data and their nuclear localization obtained after microinjection are specified.

Figure 7. Peptides derived from the putative DNA binding domain of human p27SRY show differential nuclear localization. Peptides from the DNA binding domain of human p27SRY were coupled to rabbit IgG. After purification, peptide–protein complexes were microinjected into the cytoplasm of HS68 cells. After 30 min cells were fixed and the localization of the rabbit IgG detected by indirect immunofluorescence with anti-rabbit biotinylated and fluorescent strepavidin. Shown are immunofluorescence micrographs revealing the localization of the injected peptides. (A) SRY80 peptide coupled to rabbit IgG; (C) SRY64 peptide coupled to rabbit IgG; (B and D) Inert marker antibody used to identify the injected cells. Bar, 5 μm.
from the presence of the NH2-terminal part of the HMG box. Assuming that nuclear translocation of p27SRY was carried out by the usual pathway, we analyzed the amino acid sequence of p27SRY and found a bipartite NLS in the NH2-terminal part of the HMG box. Briefly, two basic domains separated by 12 amino acids (KRxxxxxxRxxRRxK), as first described for nucleoplasmin (Dingwall et al., 1988; Dingwall and Laskey, 1991) were delineated in this part of the protein. This putative NLS present in the NH2-terminal of the HMG box of p27SRY must be deleted in the case of the truncated SRY64 peptide.

Microinjection of Peptides Reproducing the Putative p27SRY Nuclear Localization Signal

To confirm the role of a putative NLS, a 21-amino acid peptide (Fig. 6) corresponding to the NH2-terminal part of the p27SRY HMG box was synthesized and coupled to rabbit IgG (SRY21-IgG). As for SRY80-IgG and SRY64-IgG, SRY21-IgG was injected in the cytoplasm of HS68 cells and processed for immunofluorescence (see Materials and Methods). Furthermore, the SRY17 peptide derived from the NH2-terminal part of full-length p27SRY protein (Fig. 6) and carrying no putative nuclear localization sequence was coupled to rabbit IgG (SRY17-IgG) and used as a negative control. Finally, as a positive control, we produced an IgG-coupled peptide reproducing the strong nuclear localization signal from SV-40 large T antigen (Dingwall et al., 1991). As shown in Fig. 8 (A and B), this SRY derived peptide SRY21 is able to drive rabbit IgG to the nucleus by itself, in the same way as the SV-40 NLS peptide (Fig. 8, E and F). On the contrary, the SRY17 peptide is inefficient for such a function (Fig. 8, C and D). Finally, in cells directly injected with rabbit IgG, only cytoplasmic staining can be detected (data not shown).

We can conclude from these experiments that the NH2-terminal region of the DNA binding domain of p27SRY contains NLS.

Mutational Analysis of the p27SRY NLS

To further investigate the importance of the p27SRY NLS, we examined the subcellular localization of the full-length protein deleted of the 20 first amino acids in its HMG domain.

Deletion of amino acids 4Asp to 78Met was performed in pJ3SRY expression vector (termed pJ3SRYΔNLS) as described in Materials and Methods. HS68 cells were fixed 12–15 h after microinjection of the resulting plasmid. Anti-p27SRY staining revealed a bright immunoreactivity of...
the cytoplasm, demonstrating that p27SRY nuclear transport is controlled by the first 20 amino acids of the HMG domain (Fig. 9 A, p27SRYΔNLS staining; B, marker antibody). In cells harboring a strong expression of p27SRYΔNLS, a little nuclear staining was also observed (data not shown). To hypotheses can be made: first, another NLS of lower efficiency is present elsewhere in the p27SRY protein. Second, considering that the SRY gene product is a relatively small protein (27 kD), it is not surprising to observe a passive diffusion of the NLS deleted protein into the nucleus across nucleopores (Peters, 1984, 1986). This diffusion was confirmed by the use of wild type p27SRY and p27SRYΔNLS fused to the β-galactosidase gene in a pCH110 plasmid (an SV-40 expression vector containing the β-galactosidase gene). After microinjection and immunofluorescence detection with the Y127.3 antibody, the p27SRYΔNLS-β-galactosidase fusion protein was only localized in the cytoplasm, in contrast with p27SRY-β-galactosidase which was nuclear (data not shown).

Only mutation of both basic clusters can distinguish a bipartite NLS from a SV-40 prototype, i.e., a monopartite NLS. To complete these data, the two basic clusters were mutagenized independently to show their interdependence. In the first mutant (termed pJ35SNLM1), amino acids 61Lys-62Arg were changed for Ser-Leu. In the second one (termed pJ35SNLM2), amino acids 75Arg-76Arg were changed for Leu-Leu. Each expression vector was injected into HS68 growing cells as described before. As shown in Fig. 9 (C, p27SRYNLM1 staining; D, marker antibody; E, p27SRYNLM2 staining; F, marker antibody), the two mutant proteins display nuclear transport dramatically reduced or even completely abolished. Taken together, these data suggest that the p27SRY NLS requires both basic motifs in their integrity to allow the nuclear transport of the p27SRY protein. To conclude, these results are consistent with p27SRY protein nuclear localization under control of a single bipartite NLS located in the NH2-terminal part of the HMG domain.

**Discussion**

To gain insights into the mode of action of the human sex determining protein p27SRY, we have analysed its subcellular localization in mammalian cells using Western immunoblot-

**Figure 9.** p27SRY nuclear transport is abolished by deletion and mutation of the NLS present in the NH2-terminal part of the HMG domain. Plasmids encoding either a deleted NLS or mutated form of the p27SRY protein were injected into HS68 cells. After 12–15 h at 37°C, cells were fixed and localization of the p27SRY protein determined by indirect immunofluorescence with the anti-SRY antibody Y127.3. Immunofluorescence micrographs show: (A) the localization of p27SRYΔNLS protein harboring a deletion of amino acids 58Asp to 70Met, (B) insert marker antibody to reveal the injected cells with plasmid pJ35SNLS, (C) the localization of mutant p27SRY NLM1 where amino acids 61Lys-62Arg were changed for Ser-Leu, (D) marker antibody co-injected with the plasmid pJ35SNLM1, (E) the localization of mutant p27SRY NLM2, where amino acids 75Arg-76Arg were changed for Leu-Leu, and (F) marker antibody co-injected with the plasmid pJ35SNLM2. Bar, 5 μm.
Nuclear localization of p27SRY in cells expressing this protein transiently differs from that of cells overexpressing the protein transiently. In NT2D1 or H8.2 cells as well as in fetal gonadal tissue, the protein localizes to a discrete nucleus. The same result is obtained when NT2D1 or H8.2 cells are microinjected with the pJ3SRY expression vector (data not shown). It is clear that microinjected cells contain many copies of the expression vector, hence, drowning with the p27SRY protein, resulting in homogeneous staining. However, in stably transfected HeLa cells with the pJ3SRY expression vector, nuclear staining is homogeneous and cannot be compared with p27SRY staining observed in NT2D1 or H8.2 cells. Thus, p27SRY could require a particular cellular environment to display punctated pattern as observed in NT2D1 or H8.2 cells. This precise localization could represent the binding of p27SRY to specific DNA target sequences within the nucleus. The p27SRY protein has been shown to bind to the DNA in a sequence specific manner using small target sequences such as AACAAAG (Harley et al., 1991) or ACAAT (Denny et al., 1992). These small DNA motifs must be present in a high copy number in the human genome. However, since only 40 to 100 immunofluorescence foci are revealed with the Y127.3 antibody, criteria other than intrinsic DNA binding specificity must be taken into account for this p27SRY protein localization. One of them could be an association of the p27SRY protein with other transcription factors, themselves interacting with their own DNA target sequences, as recently suggested for the HMG box containing protein termed LEF1 (Giese and Grosschedl, 1993). Furthermore, the specificity of p27SRY DNA binding could be increased by interaction with a species specific protein complex, as determined for another HMG domain containing protein, UBF (Bell et al., 1990).

Table I. Comparison of the Human p27SRY Nuclear Localization Signal with Putative Nuclear Localization Signals of SOX and HMG Box Containing Proteins

| Species          | Nuclear Localization Signal | References |
|------------------|-----------------------------|------------|
| Human SRY        | KRpmnaflwvRdgRRK            | (Sinclair et al., 1990) |
| Mouse Sry        | KRpmnaflwvRgeRhK            | (Gubbay et al., 1990)   |
| Rabbit SRY       | KRpmnaflwvRqRRq             | (Sinclair et al., 1990) |
| Dunnart (marsupial) SRY | KRpmnaflwvRqRRq           | (Foster et al., 1992)   |
| Gorilla SRY      | KRpmnaflwvRqRRq             | (Whitfield et al., 1990) |
| Mouse sox-1      | KRpmnaflwvRqRRq             | (Gubbay et al., 1990)   |
| Mouse sox-2      | KRpmnaflwvRqRRq             | (Gubbay et al., 1990)   |
| Mouse sox-3      | KRpmnaflwvRqRRq             | (Gubbay et al., 1990)   |
| Mouse sox-4      | KRpmnaflwvRqRRq             | (Gubbay et al., 1990)   |
| Mouse sox-5      | KRpmnaflwvRqRRq             | (Denny et al., 1992)    |
| Human TCF-1      | KRpmnaflwvRqRRq             | (Van de Wetering et al., 1991) |
| Rat IRE-ABP      | KRpmnaflwvRqRRq             | (Nasrin et al., 1991)   |
| Human HMG1/2     | KRpmnaflwvRqRRq             | (Wen et al., 1989)      |
| Mouse LEF-1      | KRpmnaflwvRqRRq             | (Travis et al., 1991)   |
| Schizosaccharomyces Pombe Mc | KRpmnaflwvRqRRq | (Kelly et al., 1988) |

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protein: a well-documented DNA binding activity, and nuclear localization, as shown precisely in this manuscript. Interestingly, these two key functions are specified by the only conserved motif of the SRY gene throughout mammalian evolution: the HMG domain.

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